

**FOOD INNOVATION  
FOR ASIAN COMMUNITY  
DEVELOPMENT**



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# **FOOD INNOVATION**

**FOR ASIAN COMMUNITY DEVELOPMENT**

## PROCEEDINGS OF THE 3rd INTERNATIONAL CONFERENCE ON SUSTAINABLE GLOBAL AGRICULTURE AND FOOD

**09 - 10 November 2018, Ho Chi Minh City, Vietnam**



**SCIENCE AND TECHNICS PUBLISHING HOUSE**





# PREFACE

The International Conference on Sustainable Global Agriculture and Food (ICSAF) is an important event organized every two years. The 3<sup>rd</sup> ICSAF was held in Ho Chi Minh City, Vietnam, under the auspices of Vietnam Association of Food Science and Technology (VAFOST). Saigon Technology University, Vietnam (STU) was Organizer, and Assumption University, Thailand (AU), Fu Jen Catholic University, Taiwan (FJCU), Soegijapranata Catholic University, Indonesia (SCU) were Co-Organizers of this conference. The theme was “**Food Innovation for Asian Community Development**”.

The purpose of the Conference was to highlight significant research and developments for sustainable global agriculture and food production with an emphasis on product innovation. The Conference also aimed to advance regional cooperation in order to promote and apply the research in food science and technology. Aside from food technology development and food innovation, the conference also discussed issues related to Nutrition, Sensory Science, Food Science, Food Engineering and Technology, Food Safety and Quality. This Proceedings will cover all of these discussions.

The Proceedings is divided into 4 sessions covering a wide range of topics of food science and technology. Furthermore, issues related to agriculture, biotechnology, and the environment are also addressed in line with the theme of the Conference.

The papers have been peer-reviewed and edited by an editorial board of the Organizing Committee to be as readable as possible.

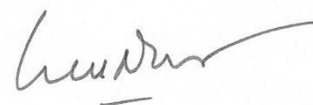
The Organizing Committee wishes to thank all authors and delegates, and all who have contributed to the success of the Conference.



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**Prof. Dr. Luu Dzuan**  
Dean of Food Technology Faculty  
(STU)



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9-10 November 2018, Ho Chi Minh City, Vietnam



#### **Conference Organizers**

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**Assumption University, Thailand (AU)**  
**Fu Jen Catholic University, Taiwan (FJCU)**  
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## **SESSION 1**

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# **NUTRITION, SENSORY, PACKAGING AND FOOD DEVELOPMENT**



## **GEARING TOWARDS COMMUNITY SERVICE SUCCESS CASES: FROM LABORATORY BENCH TO SOCIETY**

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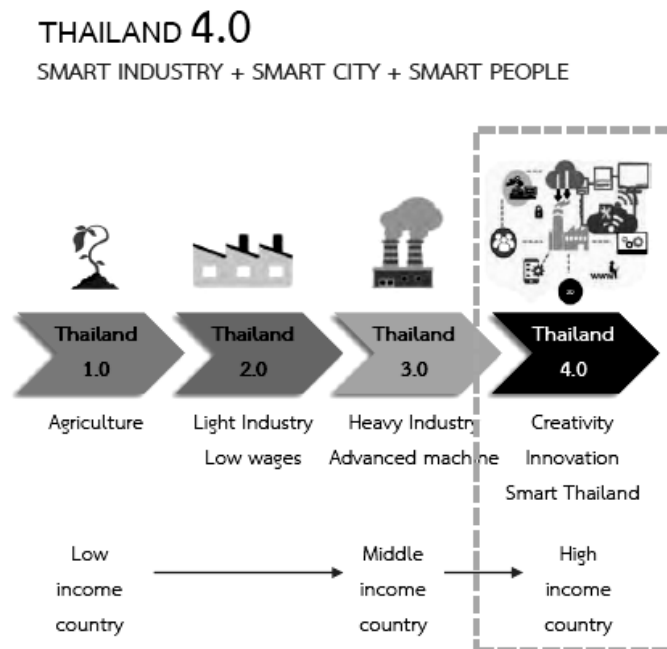
### **ABSTRACT**

Thailand 4.0 attempts to transform the country's economy from one reliant on manufacturing existing products designed by others to one driven by innovation, research and development. The role of research institutes and universities is to help local manufacturers to meet both national and international food standards by providing assistance in research and development. Besides that, food technology and biotechnology as well as innovation is also reinforcing the sustainability by assisting product development, packaging design, shelf-life stability, processing technology and food safety in parallel with Government's policy. Faculty of Biotechnology, Assumption University plays a role in driving forward the innovative enterprise sector for the Thai society to facilitate innovation systems based on the use of appropriate knowledge and technology. Actual implementation and community involvement are expected to provide the communities and startup businesses with revolutionary innovation. An example of success story was "Baan Phetplerndin Project" that Community Development Department, Ministry of Interior also gave the financial support to use research in Food Technology and Biotechnology to develop 25 valued products from 25 communities in 4 provinces. The community had the problems of unstable quality, shelf-life stability and safety of these traditional products. Faculty of Biotechnology came in to establish simple and appropriate products and process development as well as market channels by using science, technology and innovation processes. Now, their products have markedly increased in volume of sales and number of products as well as worldwide markets. The product development process was done either from community needs to the laboratory (class or special project) or from research shelf to serve community needs. The new products have increased in value more than 35%. Not only increase the product value but community service allows students to learn more about their personal motivations, practice academic material outside of the context of the classroom and testing, develop critical thinking skills while solving real-world problems, and to think about problems and social issues in new ways.

**Keywords:** Product, Community service

## 1 THAILAND 4.0

Thailand 4.0 is an economic model that aims to unlock the country from several economic challenges resulting from past economic development models which place emphasis on agriculture (Thailand 1.0), light industry (Thailand 2.0), and advanced industry (Thailand 3.0) [1]. These challenges include “a middle income trap”, “an inequality trap”, and “an imbalanced trap”.



**Figure 1:** Thailand 4.0 overview

The four objectives of Thailand 4.0 [2]

1. **Economic Prosperity:** To create a value-based economy that is driven by innovation, technology and creativity. The model aims to increase Research and Development (R&D) expenditure to 4% of GDP, increase economic growth rate to full capacity rate of 5-6% within 5 years, and increase national income per capita from 5,470 USD in 2014 to 15,000 USD by 2032.
2. **Social Well-being:** To create a society that moves forward without leaving anyone behind (inclusive society) through realization of the full potential of all members of society. The goals are to reduce social disparity from 0.465 in 2013 to 0.36 in 2032, completely transform to social welfare system within 20 years and develop at least 20,000 households into “Smart Farmers” within 5 years.
3. **Raising Human Values:** To transform Thais into “Competent human beings in the 21st Century” and “Thais 4.0 in the first world. Measures under Thailand 4.0 will raise Thailand HDI from 0.722 to 0.8 or the top 50 countries within 10 years, ensure that at least 5 Thai

universities are ranked amongst the world's top 100 higher education institution within 20 years.

4. Environmental Protection: To become a livable society that possesses an economic system capable of adjusting to climate change and low carbon society. The targets are to develop at least 10 cities into the world's most livable cities, reduce terrorism risk, and increase the proportion

## **2 NATIONAL STRATEGY: DEVELOPMENT OF TECHNOLOGY CLUSTER AND FUTURE INDUSTRIES**

In order to transform Thailand's comparative advantage into competitive advantage through knowledge, technology, and innovation, a long term goal to develop 10 Future Industries (First S-Curve and New S-Curve) has been set by the government [3].

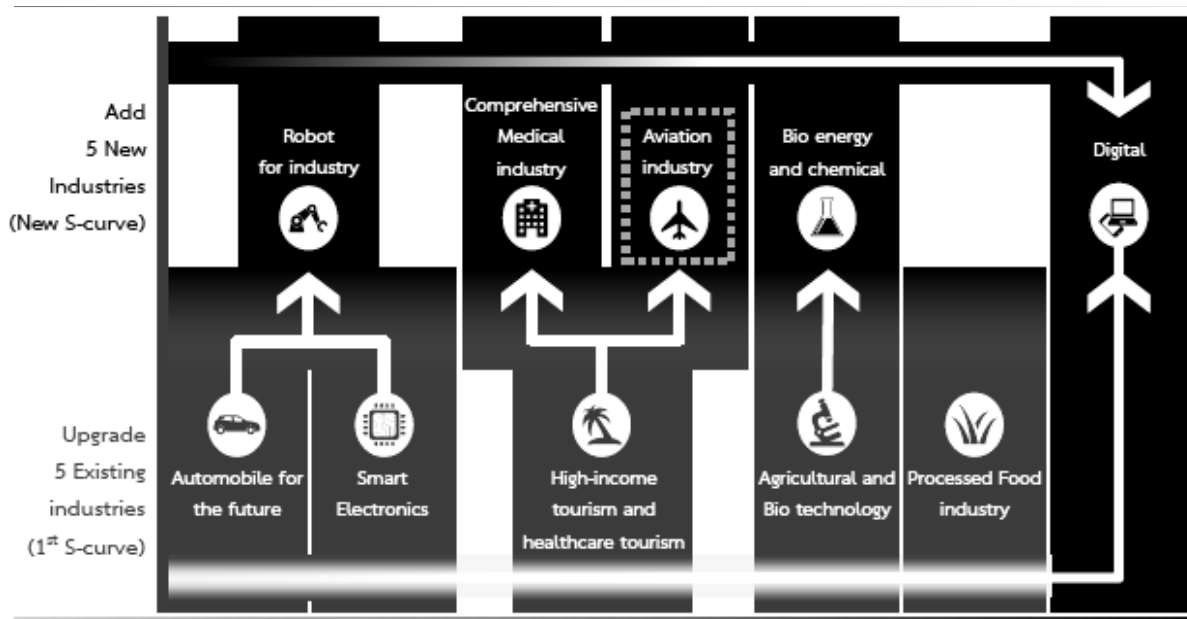
The First S-Curves was building upon 5 old industries that already have solid foundation but still require further innovative improvement and research and development to add value and keep up with competition in the global field (Next generation Automotive, Smart Electronics, Affluent, Medical and Wellness Tourism, Agricultural and Biotechnology, Food for the Future) [2].

The New S-Curves was developing the 5 new industries to enhance their capabilities to support future competitiveness (Robotics, Aviation and Logistics, Biofuels & Biochemical, Digital, Medical Hub) [2].

This approach will fulfill Thailand with knowledge, creativity, innovation, science, technology, research and innovation, and build upon comparative advantage with "5 Groups of Technology and Targeted Industries" which comprises of [2]:

1. Food, Agriculture and Biotech: building a stable economy base on biodiversity and environmentally friendly biotechnology and moving Thailand toward becoming the center of premium agricultural products and food, and an exporter of technology in agriculture, seeds, vaccine.
2. Health, Wellness and Biomedical: building medical infrastructure and move Thailand forward to be "Medical Hub" of ASEAN within 2025.
3. Smart Devices and Robotics – Mechatronics: moving Thailand forward to advance as a leader in automatic system, industrial robotics, and service robotics in ASEAN.
4. Digital, Internet of Things (IoT), Artificial Intelligence and Embedded Technology: using digital tools and IoT as platforms to enhance productivity, quality and innovation in various economic activities within agriculture, industrial, service and education sectors.
5. Creativity, Culture and High-Value Services: undertaking actions that synergize basic cultural assets, innovation and technology in order to increase commercial value and ultimately enable

Thailand to move forward in becoming one of ASEAN's "Creative hubs" within the next ten years.



**Figure 2:** The transformation between the first S-curve and the new S-curve of Thailand [4]

The "5 technology and targeted industries" will be transformed into "integrated research" in order to provide possible solutions to challenges that may arise at the national and global levels, as well as identify business opportunities for the private. At the initial stage, the government will pursue the following five agendas [2]:

Integrated research on;

1. Food and Agriculture
2. Energy
3. Aging Societies
4. Smart Cities
5. Creative Economy

### **3 SUCCESS CASE: FROM LABORATORY BENCH TO SOCIETY**

#### **"Baan Phetplerndin Project"**

Faculty of Biotechnology, Assumption University plays a role in driving forward the innovative enterprise sector for the Thai society to facilitate innovation systems based on the use of appropriate knowledge and technology. Actual implementation and community involvement are expected to provide the communities and startup businesses with revolutionary innovation.



An example of success story was “Baan Phetplerndin Project” that Community Development Department, Ministry of Interior also gave the financial support to use research in Food Technology and Biotechnology to develop 25 valued products from 25 communities in 4 provinces. The community had the problems of unstable quality, shelf-life stability and safety of these traditional products. Faculty of Biotechnology came in to establish simple and appropriate products and process development as well as market channels by using science, technology and innovation processes. Now, their products have markedly increased in volume of sales and number of products as well as worldwide markets.

The product development process was done either from community needs to the laboratory (class or special project) or from research shelf to serve community needs. The new products have increased in value more than 35%. The approximate value was 25,000,000 Baht. Not only increase the product value but community service allows students to learn more about their personal motivations, practice academic material outside of the context of the classroom and testing, develop critical thinking skills while solving real-world problems, and to think about problems and social issues in new ways.



Tom Yum Chakham crispy baby clam



Banana powder Drink



Toddy palm soap



Crispy waffle - Sweet mango sauce coating



Lime oil mouthwash tablet



Low GI soymilk panna cotta



Ready to eat steamed Mackerel in retort pouch



Pineapple rice cracker



Coconut oil shampoo-conditioner bar



Spa aroma salt



Pineapple sauce



Low GI Thai shortbread cookies



Honey-lime pressure infused young toddy palm



Shrimp paste sauce

### 3 CONCLUSIONS

The success of Thailand's in promoting the creative and innovative society of Thailand 4.0 require research and knowledge transferring by creating a network alliance to propel basic and applied research to national and international level, putting a research fund for innovation development in place to continue transnational research, allowing tax exemptions on the import of materials for research. Academic institute need to play as the research and knowledge hub to provide all scientific needs of SMEs and community in order to increase GDP and GDH of Thailand.

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## **EFFECT OF PACKAGING MATERIALS ON QUALITY CHANGES DURING STORAGE OF NONSEE-PEEL CURED JELLYFISH**

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### **ABSTRACT**

Effects of packaging types on turbidity, color, pH and texture of the Nonsee-peel cured jellyfish were studied to consider the suitable type of packaging. The packagings used were regular polypropylene plastic bags (PP), 230-mL PP cups, vacuum bags (PET) and biodegradable bowls (PLA/PP). The proximate analysis of Nonsee peel cured jellyfish was also conducted to perceive the profile of the product. The results showed that the moisture of jellyfish was approximately 98% in both bell and arm parts while protein and fat contents were found to be significantly ( $p \leq 0.05$ ) higher in the bell, 0.78% and 0.86%, respectively. There were significant differences in turbidity, color and pH between packaging types over two weeks storage at ambient temperature ( $p \leq 0.05$ ). The bell jellyfish packed in the plastic PP cup had the least total color difference ( $\Delta E^* = 4.8281$ ). However, there was no significant difference in firmness, toughness and springiness during storage days of the bell jellyfish packed in the plastic PP cup ( $p > 0.05$ ). Hence, the plastic PP cups are anticipated as a proper packaging that could maintain the color and texture changes of the Nonsee peel cured jellyfish.

**Keywords:** cured jellyfish, jellyfish, Nonsee-peel cured, PP cup, packaging

### **1 INTRODUCTION**

Jellyfish is one of the marine animals claiming to contain high amount of collagen [1]. Collagen is renowned as a bioactive ingredient known for its beauty-aid revitalizing. Biomedical applications of the collagen can help maintaining healthy skin, curing wounds or burns, reconstructing of vascular and cardiac as well as helping in cartilage and bone reconstruction [2]. Main sources of the collagen are from skin and bone of cow and pig. Other animal sources include chicken, sheep skin, kangaroo tail, horse tendon, fish, other marine animals, etc.

The jellyfish are cured and prepared for consumption in various dishes in Asian food. For curing techniques, there are two different solutions mainly used for jellyfish preservation, nonsee peel solution and alum-salt solution. Nonsee peel cured jellyfish is the native food product from Eastern provinces in Thailand, e.g. Trad and Chuntaburi. The jellyfish are cured by using Nonsee bark solvent, resulting in dark red-color jellyfish from the color of the bark. The bark has good

ability in reducing the fishy smell of the jellyfish [3]. It is normally eaten with the local special recipe dip. However, this product is only available in the locals, well-known due to the packaging that the producers currently use. Regular polypropylene bags have been using as the container for the product. With the short shelf-life storage, as well as poor protection of the jellyfish products in term of packaging and transportation, it is difficult for the products to be distributed widely throughout the country.

Thus, the study of appropriate type of packaging for the Nonsee-peel cured Jellyfish would provide valuable benefit to both producers and consumers. The proper packaging will help add value to the local fishery product and make this product to be more recognizable to the market. Currently, there is no study about the proper packaging materials acquiring for a longer shelf-life of the Nonsee-peel cured jellyfish product. Therefore, this study aims to examine the proximate analysis of Nonsee peel cured jellyfish and the effect of different types of packaging to the shelf-life of the product.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Nonsee peel cured jellyfish were obtained from Trad province in Thailand. The regular plastic bag (polypropylene) 5''×8'' (PP), vacuum bags 5''×8'' made from polyethylene terephthlate (VB), 230 mL of plastic PP cups (No.1641) (PC) and biodegradable bowls made from polylactic acid and a minute amount of polypropylene (BB) used for storage test were purchased from local stores.

### **2.2 Proximate analysis of Nonsee peel cured jellyfish**

The Nonsee peel cured jellyfish shipped from Trad were packed in the plastic bag with regular tap water and stored at 4°C until required. To perform an analysis, the jellyfish were moved out from the cured solution and cut into small pieces, 1-centimeter dice. The jellyfish body were distinguished into two parts which were bell and arm. Each part was prepared for analysing the moisture content, fat, ash [4], and protein content (Kjeldahl method). The experiments were run in triplicate.

### **2.3 Effect of packaging on the storage test**

#### ***2.3.1 Jellyfish preparation***

The Nonsee-peel cured jellyfish bells were rinsed under running fresh water until all particle was visually removed. The bodies were cut to separate into two parts, bell and oral arms. The bells were cut into thin strips approximately 3 mm thick. The arms were cut in cross sections



approximately 3 mm thick. Then, the cut jellyfish were soaked in fresh water before packing to prevent moisture loss.

### *2.3.2 Effect of packaging on the ambient storage*

There were 4 types of packaging including regular plastic bag (polypropylene) 5''×8'' (PP), vacuum bag 5''×8'' (VB), 230 ml of plastic PP cup (No.1641) (PC) and biodegradable bowl (BB). Each type of container contained 40 grams of the bell, and 120 grams of soaking water. Package PB were tied with rubber bands, whereas package VB were sealed by the hot seal machine. Package PC were closed by using PP lids while package BB were sealed with food wrapper. Each container was subjected to sampling to observe the color and texture of jellyfish and pH and turbidity of the soaking water after 1, 4, 7, 12 and 14 days with ambient storage.

### *2.3.3 Measurement*

#### **Color**

Color were measured by a colorimeter measuring at 5 different positions at the bell part of the jellyfish. The arithmetic mean was calculated and also the overall color change  $\Delta E^*$  was measured using the CIE  $L^*a^*b^*$  color measuring system.  $L^*$  describes the lightness, and  $a^*$  and  $b^*$  describe the chromatic coordinates on the green-red and blue-yellow axes, respectively. The total color difference ( $\Delta E^*$ ) was calculated using the following formula:

$$\Delta E^* = [\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}]^{1/2}$$

$\Delta L^*$ ,  $\Delta a^*$ , and  $\Delta b^*$  are the differences between the initial and the final values (Day 1 and Day 14) of  $L^*$ ,  $a^*$  and  $b^*$ , respectively.

#### **Texture**

The texture of bell part was measured by texture analyzer (TA-XT plus, Charpa Techcenter Co., Ltd). Texture profile analysis (TPA) and Single-blade shear evaluation 39 were selected as methods to observe the changing of texture in hardness, firmness, toughness and springiness.

#### **Turbidity**

Two milliliters of the soaking water from each package was transferred into a cuvette and measured the turbidity by spectrometer at wavelength 600 nm to observe. The result was done in five copies.

### *2.3.4 Statistical Analysis*

The data obtained was analyzed using ANOVA and Duncan's multiple range test in SAS program at 95% confidential level.

### 3 RESULTS AND DISCUSSION

#### 3.1 Proximate analysis of Nonsee peel cured jellyfish

The majority of jellyfish composition was water, which was approximately 98 %, the rest was total solid (Table 1). Protein and fat contents were significantly high in the bell, 0.7785% and 0.86%, respectively. The study by Thumthanaruk [3] indicated different results which could be because of the difference in species used in curing [3].

**Table 1:** Proximate analysis of Nonsee-peel cured jellyfish

Part	Composition (%)				
	Total solid	Moisture	Protein	Fat	Ash
Bell	1.4876±0.22 <sup>a</sup>	98.5124±0.22 <sup>a</sup>	0.6528 ±0.10 <sup>b</sup>	0.0175±0.00 <sup>b</sup>	0.0332±0.02 <sup>a</sup>
Arm	1.7623±0.44 <sup>a</sup>	98.2377±0.44 <sup>a</sup>	0.7785±0.07 <sup>a</sup>	0.0246±0.00 <sup>a</sup>	0.0209±0.01 <sup>a</sup>

**Remarks:** - The results were analyzed by ANOVA using *t*-test

- Means of the same composition with same superscript indicating no significant difference between parts of the jellyfish ( $p>0.05$ ).

#### 3.2 Types of packaging and quality changes during ambient storage

The pH of curing solutions in all packaging were stable throughout the 2-week test. The products were not acidic food since the pH is above pH 4.6 (Table 2).

**Table 2:** Average pH value of soaking water from each packaging during two weeks storage

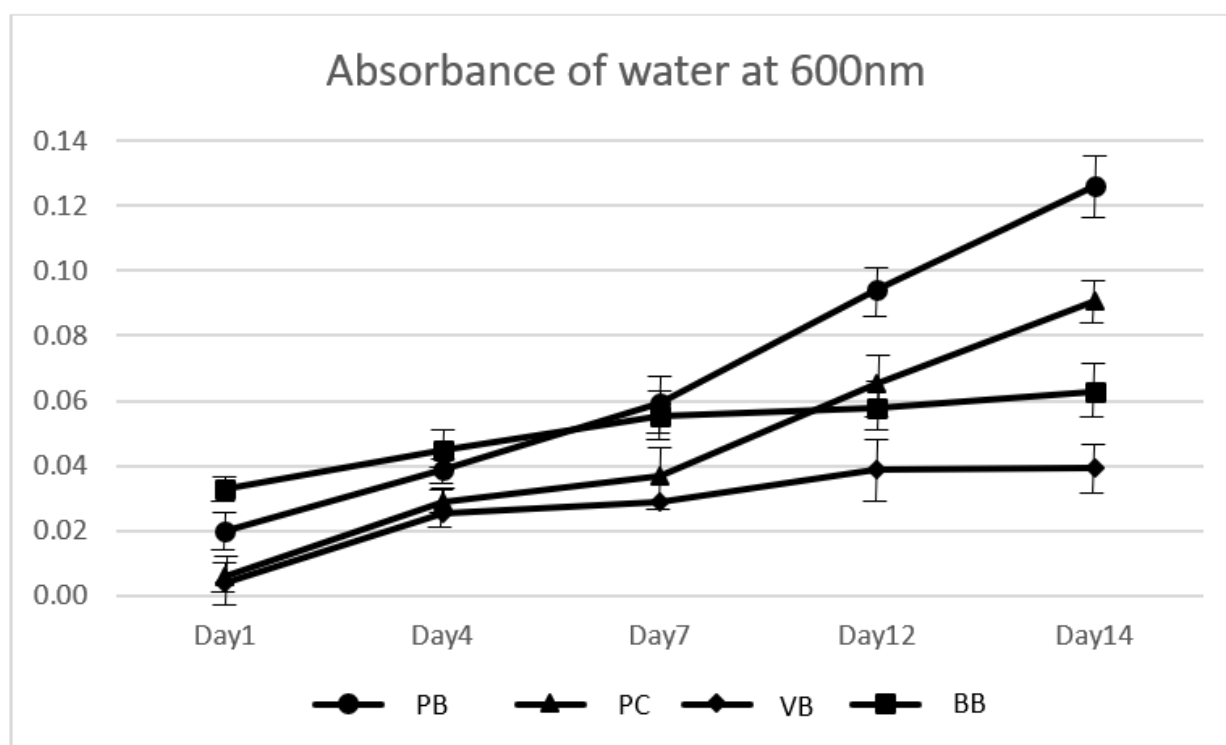
Type of packaging	pH				
	Day 1	Day 4	Day 7	Day 12	Day 14
PB	6.1240 ± 0.01 <sup>Bc</sup>	6.1260 ± 0.01 <sup>Bc</sup>	5.7920 ± 0.01 <sup>Bd</sup>	6.1880 ± 0.01 <sup>Bb</sup>	6.2700 ± 0.02 <sup>Ba</sup>
PC	5.8880 ± 0.01 <sup>Dc</sup>	5.8920 ± 0.01 <sup>Ca</sup>	5.4980 ± 0.02 <sup>Dd</sup>	6.0640 ± 0.01 <sup>Cd</sup>	5.6060 ± 0.01 <sup>Db</sup>
VB	5.9040 ± 0.01 <sup>Cb</sup>	5.7880 ± 0.01 <sup>Db</sup>	5.5460 ± 0.01 <sup>Cd</sup>	5.5080 ± 0.01 <sup>Da</sup>	5.6840 ± 0.01 <sup>Cc</sup>
BB	6.7480 ± 0.01 <sup>Aa</sup>	6.8000 ± 0.01 <sup>Ab</sup>	6.5960 ± 0.02 <sup>Ad</sup>	6.6160 ± 0.02 <sup>Ae</sup>	6.7740 ± 0.01 <sup>Ac</sup>

**Remarks:**

- The results were analyzed by ANOVA using Duncan test.

- The same letter (uppercase) in each day (column) means no significant difference between types of packaging.

- The same letter (lowercase) in each type of packaging (row) means no significant difference between days.



**Figure 1:** Turbidity of soaking water of jellyfish over storage time

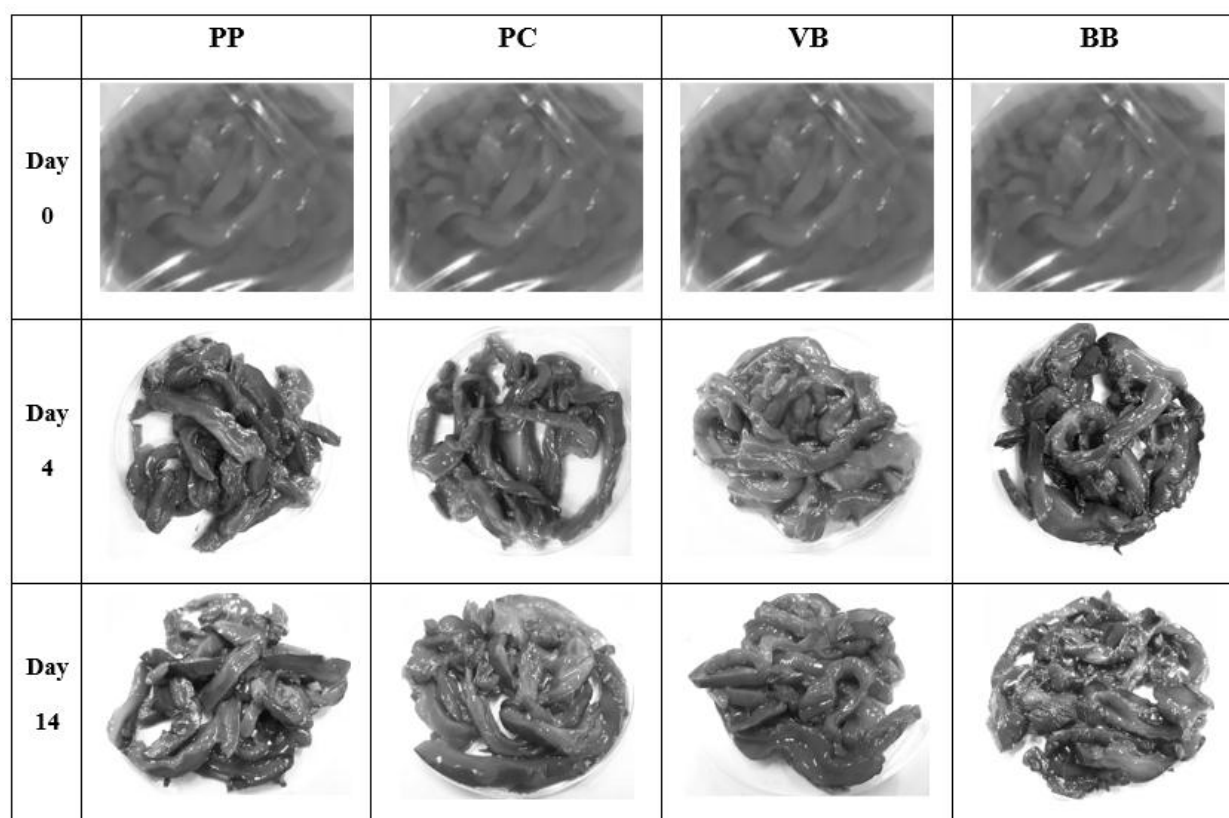
The turbidity of soaking water increased over time in all types of packaging (Fig. 1). At the end of the storage, jellyfish packed in the air seal vacuum bag had the lowest OD<sub>600</sub> value (0.0392) while one in the polypropylene bag showed the highest (0.1262) OD<sub>600</sub>. The storage time and types of packaging significantly affected the turbidity of the water ( $p \leq 0.05$ , data not shown). Jellyfish and packaging decomposition could distribute to the increase in turbidity as well as the interactions between the soaking water and package.

Color changes in the jellyfish were determined by observing the total color differences ( $\Delta E^*$ ) which are presented in table 3. The Jellyfish packed in the PP cup (PC) showed the least total color difference of the jellyfish bell part ( $\Delta E^* = 4.8281$ ) between day1 and day14. The darkness of the jellyfish could be caused by tannin and phenolic compounds from the Nonsee peel with nitrogen present in jellyfish and the packaging materials [5],[6] (Fig. 2). Another reason could be the effect of oxygen transmission rate in different types of packaging.

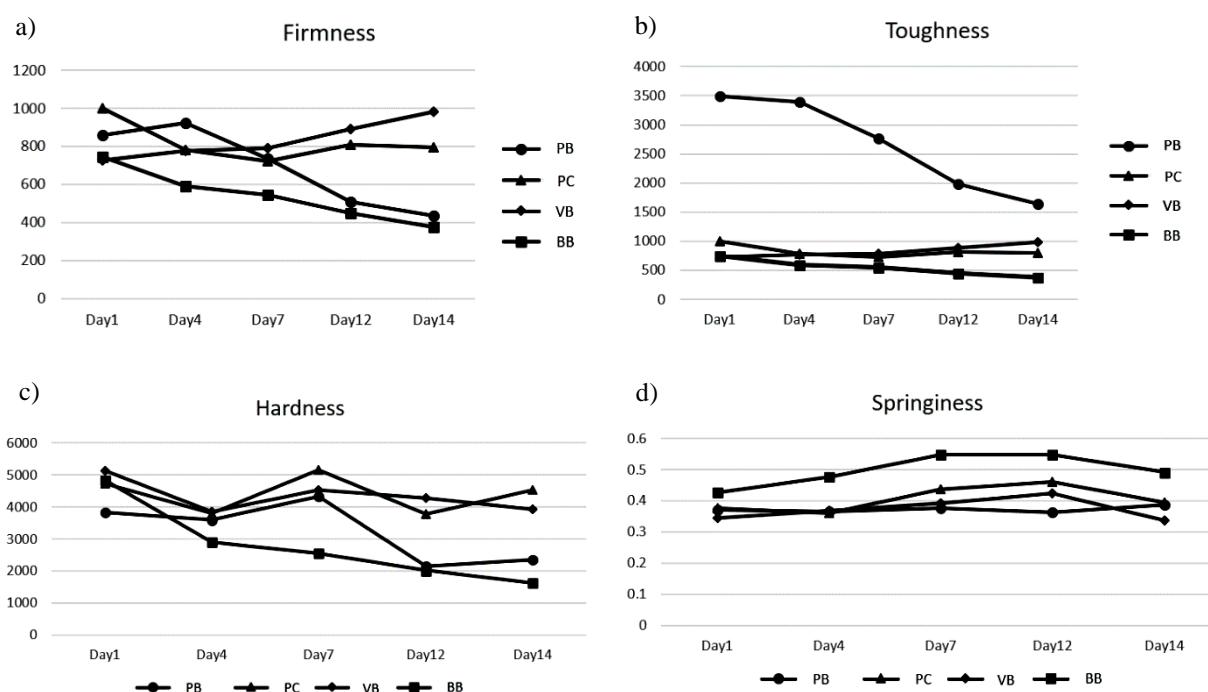
**Table 3:** Overall color changes of soaking water and bell part

	Jellyfish bell			
	PP	PC	VB	BB
$\Delta E^*$	6.8203	4.8281	16.0937	11.2726





**Figure 2:** Color changes in Nonsee-peel cured jellyfish in different types of packaging after 2 weeks of storage



**Figure 3:** Textural changes in Nonsee-peel cured jellyfish during 2-week storage under different types of packaging (a) Firmness (b) Toughness (c) Hardness and (d) Springiness

Textural changes are one of the concerns in the shelf-life of cured jellyfish. Fig. 3 shows the trends in different attributes of textural changes include firmness, toughness, hardness and springiness of the Nonsee-peel cured jellyfish. But to justify which packaging is suitable for the product, one should consider in overall changes (Table 4). The Nonsee-peel cured jellyfish packed in polypropylene cup (PC) exhibited the minimum textural changes in firmness, toughness and hardness.

**Table 3:** Overall textural changes in Nonsee-peel cured jellyfish stored from day1 to day14

Type of packaging	Firmness	Toughness	Hardness	Springiness
PP	-423.292	-1855.330	-1465.00	0.0172
PC	-204.597	-944.573	-203.22	0.0191
VB	257.135	965.883	-1190.53	-0.0066
BB	-367.607	-1544.560	-3206.25	0.0659

Remark: the (-) means the value decrease over storage time

Changes in turbidity, color, and texture suggested that polypropylene cup provided a rigid package suitable for prolonging the shelf-life of Nonsee-peel cured jellyfish. The vacuum seal bag could also be used but the textural changes might affect the shelf life.

When the Nonsee-peel cured jellyfish were packed regardless of packaging types, the quality of the jellyfish was less appealing due to the physiochemical changes of the product. To minimize these changes, product should be vacuum packed in plastic materials with a low or very low oxygen transmission rate (OTR) and water vapor transmission rate (WVTR). It is also crucial that the thickness of the film or laminate is specified for the test. Further investigation on these parameters is recommended on better understanding of the quality of Nonsee-peel cured jellyfish as affected by the packaging materials.

## 4 CONCLUSIONS

Water of jellyfish is the main composition which was approximately 98 %. Remainders include protein (0.6-0.7 %) and trace amount of fat and ash. Comparing between the parts, arm part got higher percentage than the bell for both protein and fat contents. Packaging types had effect on turbidity, color, pH, and texture of the product. The polypropylene cup was recommended as the most appropriate packaging type for better shelf-life of the Nonsee-peel cured jellyfish product. The vacuum seal bag package would also be recommended regardless of textural changes. Further investigation on oxygen transmission rate (OTR), water/vapor transmission rate (WVTR) and others is recommended for the better insight.

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## **IN VIVO NUTRITIVE VALUES OF PROTEIN EXTRACTED FROM BRACKISH GUTWEED *ENTEROMORPHA* SP.**

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### **ABSTRACT**

Gutweed (*Enteromorpha* sp.), a species of green brackish algae, distributes with a high biomass in extensive shrimp ponds in Mekong Delta. There were few publications on using them for aquaculture and food industry. This kind of algae can be further exploited as a source of protein due to its high content (13-22% w/w db). Nutritive value of protein of *Enteromorpha* sp. was determined by measuring amino acids contents by HPLC method and calculating AAS (amino acid score) of 9 essential amino acids. Other nutritive values such as BV (Biological Value), PER (Protein Efficiency Ratio), NPR (Net Protein Ratio) and PDCAAS (Protein Digestibility Corrected Amino Acid Score) were determined by *in vivo* trials on white mice (mice). These mice were fed by test diets containing *Enteromorpha* sp. protein (10%) and data were collected after 10- 20 days. BV of *Enteromorpha* sp. protein was 65.19 (72.14 in control soya protein). PER, NPR and PDCAAS were 1.67, 6.56 and 52.15 or 58.67 respectively. They are lower than those of animal proteins but equal to or higher than those of some plant proteins.

**Keywords:** *Enteromorpha* sp, green brackish algae, *in vivo* trial, nutritive value, protein

### **1 INTRODUCTION**

Seaweed has been used and studied extensively in the field of food. As the warming of the earth increases the sea level, the brackish water areas are widened. Attached to this phenomenon is the emergence of brackish water species and large biomass production. Gutweed (*Enteromorpha* sp.), the green brackish seaweed, widely distributes with large biomass in extensive brackish shrimp ponds. The protein content in this brackish algae accounts for 13-22% of dry weight. Protein from brackish gutweed is new so, in addition to studying the extraction processes, protein also needs to be evaluated for nutritional value and the digestibility, utilization. *In vivo* nutritional evaluations are necessary because these methods have a special meaning and they are likely to be required for new protein sources if protein is to be used as a food source.

The nutritional value of the protein is the amino acid composition as well as the ability to be digested, absorbed, and converted to nitrogen in the body. These values are expressed through the *in vivo* test indices including PER, NPR, AAS, BV, PDCAAS. PER compares the gain of body weight with amount of protein intake at least 10 days. PER represents the amount of protein retained in body tissue. NPR is calculated similarly to PER but NPR is added the weight lost after experimental animals had been fed with protein free diets so NPR is a conclusive measurement of protein utilization. AAS shows the level of response to the body needs of each type of amino acid in the protein preparation. When protein is digested, it is absorbed into the blood and converted into body protein so BV values indicate the level of digestion and absorption of protein in the feed. PDCAAS is the index actually recommended by the FAO/WHO to evaluate the nutritional quality of a protein for human. It is calculated by multiplying the first AAS of essential amino acid by BV. Therefore, PDCAAS takes into account three parameters, the essential amino acid profile, the digestibility and its ability to supply essential amino acid in the amounts required by human.

In this study, nutritional value of protein from *Enteromorpha* sp. will be assessed *in vivo* and compared with soy protein, a commonly used protein.

## 2 MATERIALS AND METHODS

From brackish gutweed, protein preparation was collected by extraction with 0.1M NaOH solution for 60 minutes at 50<sup>0</sup>C [5]. The protein in the extract was precipitated by 70% saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> salt at 10<sup>0</sup>C. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was removed by dialysis tube made by cellophane with 1000kDa hole size [2]. The purity of the protein preparation is enhanced by treating it with 2% cellulase to remove carbohydrate. The protein was freeze-dried and preserved.

The protein content of the preparation was determined by the Nessler method [21] after samples were digested by concentrated sulfuric acid. The amino acid profile of the protein was determined by HPLC.

- Protein powder was used to prepare feeds for experimental mice with the nutritional composition designed according to the AIN93 formula [19].
- Experimental mice were Swiss white mice provided by the Pasteur Institute of Ho Chi Minh City. Mice were about 5-6 weeks old, 16-20g weight.
- The individuals of mice were divided into four groups, each with five individuals and separately raised.
  - + Group 1: Mice were fed with protein-free diets.
  - + Group 2: Mice were fed with protein-based diets derived from gutweed protein preparation.
  - + Group 3: Mice were fed with protein-based diets derived from soybean protein.

- + Group 4: Mice were fed with diets derived from Pasteur Institute of Hochiminh City (20.4% protein)

Experimental mice were fed about 2g of food per day. After 24h, the leftover was collected to determine the amount of food consumed during the day. Each week, mice were weighed. Faeces and urine of mice were collected daily and determined the total nitrogen content.

The data obtained from mice experiment were used to calculate following parameters:

**PER** (Protein Efficiency Ratio): the ratio of grams of protein digested per grams of body weight gained

$$PER = \frac{W}{P}$$

W: Weight gain of experimental mice after 20 days of experiment (g); P: Protein weight consumed during 20 days (g).

**NPR** (Net Protein Ratio):

$$NPR = \frac{T + G}{P}$$

T: The average weight gain of the group using protein diet after 10 days (g); G: The average weight loss of the control group using a protein-free diet after 10 days (g); P: Protein weight consumed during 10 days (g).

**BV** (Biological Value):

$$BV = \frac{I - (F + U)}{I - F} \times 100$$

I: The total amount of nitrogen consumed during 20 days (mg); F: Total amount of nitrogen excreted in the faeces during 20 days (mg); U: Total amount of nitrogen excreted in the urine during 20 days (mg).

**AAS** (Amino Acid Score):

$$AAS = \frac{A}{N}$$

A: Amount of amino acid (mg) in 1g of protein; N: The essential amino acid requirements of a 1-2 years old children/adults (mg amino acid/g protein) recommended by FAO/WHO 2007

**PDCAAS** (Protein digestibility corrected amino acid score):  $PDCAAS = \text{First AAS} \times BV$

Data processing: Experiments were repeated three times. Results are represented by mean  $\pm$  SD. Statgraphic software were used to process statistics

### 3 RESULTS AND DISSCUSSION

#### 3.1 Amino acid profile and Amino Acid Score (AAS) of protein from *Enteromorpha* sp.

After extracted with 0.1M NaOH solution followed by cellulase treatment and precipitated by ammonium sulfate, the protein content of protein powder was  $52.12 \pm 1.56\%$ . Amino acid profile of protein derived from *Enteromorpha* sp. is shown in table 3.1.

**Table 3.1:** Amino acid profile of brackish gutweed protein

Essential-Amino acid	Content (mg/g protein)	Nonessential-amino acid	Content (mg/g protein)
<b>Valine</b> *	$54 \pm 5$	Cysteine	$46 \pm 2$
<b>Isoleucine</b> *	$35 \pm 7$	Aspartic acid	$124 \pm 6$
<b>Leucine</b> *	$84 \pm 5$	Glutamic acid	$165 \pm 4$
<b>Phenylamine</b> *	$54 \pm 6$	Serine	$51 \pm 3$
<b>Threonine</b> *	$40 \pm 1$	Glycine	$49 \pm 5$
<b>Lysine.HCl</b> *	$41 \pm 3$	Proline	$30 \pm 2$
<b>Histidine</b> *	$14 \pm 2$	Alanine	$75 \pm 3$
<b>Methionine</b> *	$36 \pm 3$	Tyrosine	$45 \pm 2$
<b>Arginine</b> **	$57 \pm 4$		

\*: essential amino acid \*\*: semi-essential amino acid

Result in table 3.1 shows that, protein extracted from gutweed contains all essential and semi-essential amino acid. In addition, aspartic and glutamic acid are highest fraction of amino acid in gutweed protein. Gutweed protein also contains high level of alanine and leucine. However, histidine is the lowest fraction of amino acid. This result is similar to some amino acid profile of seaweed including green, red and brown seaweeds [11].

**Table 3.2:** Essential amino acid profile of brackish gutweed protein in comparison with FAO/WHO standard and their Amino Acid Score

Amino acid	Content (mg/g protein)	FAO/WHO reference pattern for 1-2 years age children (mg/g protein)	AAS <sup>c</sup>	FAO/WHO reference pattern for adults (mg/g protein)	AAS <sup>a</sup>
Isoleucine	35	31	1.1	30	1.2
Leucine	84	63	1.3	59	1.4
Lysine	41	52	0.8	45	0.9
Threonine	40	27	1.5	23	1.7
Valine	54	42	1.3	39	1.2
Histidine	14	18	0.8	15	0.9
Methionine + Cysteine	82	26	3.2	22	3.7
Phenylamine+Tyrosine	99	46	2.2	38	2.6
<b>EAA</b>	<b>449</b>	<b>305</b>		<b>271</b>	

EAA: Essential amino acid; ASS<sup>c</sup>: Aminoacid score calculated by theoretical standard for 1-2 years age children; ASS<sup>a</sup>: Aminoacid score calculated by theoretical standard for adults

Data in table 3.2 show that threonine, methionine, cysteine, phenylalanine and tyrosine content of gutweed protein are higher than the body needs. Lysine and histidine are two amino acids that do not meet the needs of children and adults but their contents is higher than that of corn and wheat protein. Herminia [8] also showed that the lysine content of *Enteromorpha intestinalis* seaweed was 6.5 times higher than that of corn and wheat protein. However, similar to other plant protein sources - such as hemp seed protein, AAS of lysine was about 0.6 [9] – lysine and histidine can be considered as limiting amino acid in gutweed protein. Thus, to be used as a food protein source, the protein obtained from *Enteromorpha* needs to be supplemented or combined with other protein sources rich in lysine and histidine.

### 3.2 In vivo determination of nutritional value of protein from *Enteromorpha* sp.

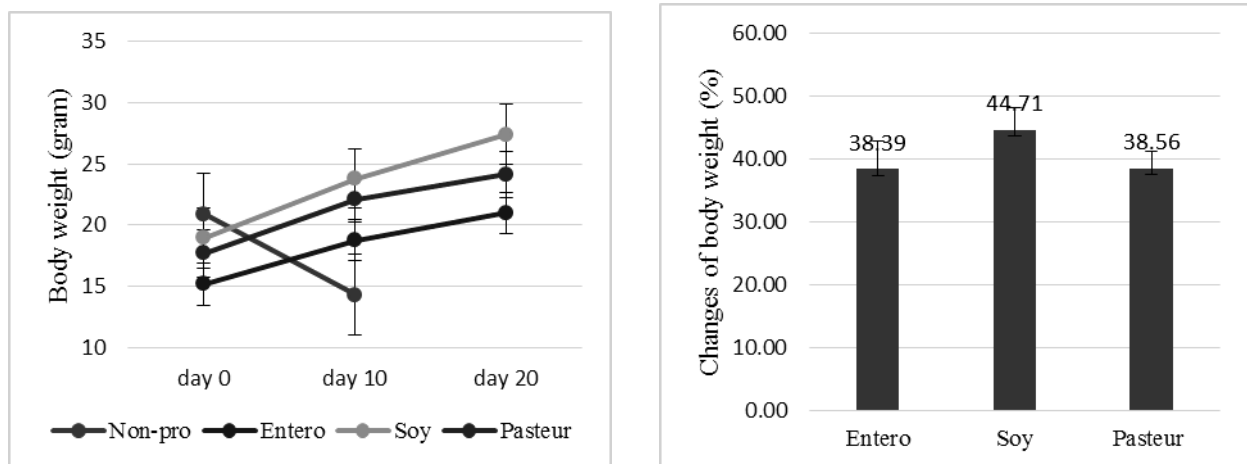
AAS only reflects inadequately the nutritional quality of the protein. Quality of protein is also expressed in the ability to be absorbed and utilized in the body. The *in vivo* experiment showed the following data:



### Changes of body weight of 4 groups of mice

Although provided with full energy diet, after 10 days using protein-free diets, the weight of the tested mice dropped sharply, at an average of 6g per individual, over 30% of the initial weight. Mice exhibited slug behavior, or lazy activity, and their furs were not as smooth as in 1-2 early days. This result confirms the role of dietary protein in the body.

Figure 3.1 also shows that when fed with adequate nutrient groups according to the AIN-93 formula, the weight of the mice were significantly increased. Mice exhibited excellent mobility and responsiveness, good behavior and smooth furs. After each week, body weight increased 2-3g (10-15% of initial weight). These changes were different in the 3 groups of tested mice. Moreover, with the same formula of diet (AIN-93) but with different protein sources, the percentage of body mass weight gain are different as well. After 20 days, mice fed diets containing soy protein increased their body weight by 44.7%, the gutweed protein feed group increased 38.39% of body weight.



**Non-pro:** non protein diet; **Entero:** Enteromorpha protein based diet

**Soy:** Soybean protein based diet; **Pasteur:** Pasteur Institute diet

**Figure 3.1:** Changes of body weight of 4 groups of mice after 20 days (g and %)

This result confirmed the role of body building of proteins and the dissimilar effects of different proteins on body. Soy protein has long been used and has been shown to have higher nutritional value compared to many other plant-derived proteins. Thus, soy protein helped mice gain weight better than mice which consumed gutweed protein diets.

### PER and NPR values

PER value of plant proteins are generally lower than that of animal proteins (usually above 2.5). For example, PER values of legume proteins are 0.9-1.8 whereas PER of casein is 3 [25]. A PER below 1.5 approximately describes a protein of low or poor quality, the value between 1.5 and 2.0 is intermediate quality and above 2.0 is good to high quality [15].

**Table 3.3:** PER and NPR values of 3 experimental proteins

Protein source	PER	NPR
<i>Enteromorpha</i> sp.	1.67	6.56
Soybean	2.35	7.30
Food from Pasteur Institute of HCM City	0.8	NK

NK: not known

The results shows that the PER of the control soybean protein was 2.35, similar to that of other studies such as 2.69 [7], 2.30 [20] The PER of the protein from *Enteromorpha* was 1.67, lower than the PER of animal proteins (3.85 in casein, 4.29 in lactalbumin [7], or some plant proteins such as 2.8 in rice, 2.6 in potato [7]. However, PER value received was much higher than the PER of some common plant proteins such as 0.95 in wheat flour [1], 0.87 in black beans [7].

The difference in the value of two indexes- PER and NPR- depends on the origins of protein. For example, NPR of lean beef was 88% higher than its PER. NPR result in table 3.3 showed the equivalent quality of gutweed protein and soybean control protein. The gutweed NPR value was 6.56, slightly smaller than the value 7.30 of the control soybean protein. Thus, the protein from gutweed is equivalent to soy in maintaining the viability of the living organism.

#### *BV and PDCAAS values*

Protein in the food is partially digested, the rest will be excreted through in the faeces. If protein of food cannot be converted to body protein, the N of their amino acids is discharged through the urinary tract. The BV index shows the efficiency of using protein for the body. When the amount of nitrogen retained by the body is not high, the BV value is very low.

**Table 3.4:** Average amount of intake nitrogen and nitrogen excreted in faeces and urine during 20 days

Group of mice	N in intaken food (mg)	N in faeces (mg)	N in urine (mg)	BV
Soybean protein-based diet	57,60 ± 0,59	9,28 ± 0,02	13,46 ± 0,06	72.14
<i>Enteromorpha</i> protein-based diet	55,84 ± 0,31	10,34 ± 0,02	15,84 ± 0,35	65.19

The BV value is often high in animal proteins which be seen in many researches. For example, Lima E Silva et al. [12] reported that the BV of casein was 87.82. BV values varies widely among plant proteins. Nishizawa et al. [18] described the protein quality of rices with BV ranging from 65-75, the BV of Carrot proteins from the research of Bruunsgaard et al [3] were 77-82. In recent researches, many new protein sources have been calculated their BV. However, these values were not high. BV of grain protein of new Amaranths varieties ranged from 44.53 to 6.28

[4], BV of root and leaves of Anchote (*Cocconia abyssinica*) were 26.76 and 47.09 respectively [24].

The BV of control soybean protein in this study is close to that of many other studies, about 70 or 80 [20]. BV value of gutweed protein was 65.19, a moderate result comparing with other plant proteins. It was lower than that of control soy protein and animal proteins but higher than that of many new plant proteins. Furthermore, the content of protein in protein preparation used for this studied was only about 50%. If its purity is increased, the BV can be higher.

**Table 3.5:** PCDAAS of brackish gutweed calculated by basing on the requirement of children and adults

Based on the requirements of children of 1-2 years age		Based on the requirements of adult	
The first AAS <sup>a</sup>	PDCAAS <sup>c</sup>	The first AAS <sup>a</sup>	PDCAAS <sup>a</sup>
0.8 (lysine/histidine)	52.15	0.9 (lysine/histidine)	58.67

The greater the value of PDCAAS is, the higher the protein's ability is to meet the requirements of amino acids. This value is so high in animal protein such as 100 in casein, egg white, 92 in beef, and in some plant protein such as 100 in soybean isolate, 73 in pea protein concentrate, 68 in kidney bean. It is so low in many plant proteins such as 52 in peanut, 25 in wheat gluten, 23 in almond [6].

The values of PDCAAS calculated for brackish gutweed were about 52-59, which are moderate values. It is not high because the first AAS value is 0.8 or 0.9 from histidine or lysine. It means that these amino acid only meet 80-90% of the body needs of children 1-2 years of age and adults. This finding suggests that histidine and lysine should be concerned when using brackish gutweed as a source of protein for human. In addition, the BV value of the gutweed protein was only 65.19, which was the second reason why the PDCAAS was not high. Thus, besides raising the purity of the protein powder to increase the value of BV, adding histidine and lysine is a potential way to improve the nutritional value of gutweed proteins.

#### 4 CONCLUSIONS

Protein derived from brackish gutweed (*Enteromorpha* sp.) contains 9 essential amino acids. There were 7 essential amino acids that are higher than the requirements of 1-2 years old children and adults. Lysine and histidine were two amino acids that meet only 80-90% of the body requirement of both adults and children. After 20 days, mice fed diets containing soy protein increased their body weight by 44.71%, the gutweed protein feed group increased 38.39% of body weight. The PER of the protein from *Enteromorpha* was 1.67, lower than that of the control soybean protein (2.35). With two tested proteins, PER and NPR of soy was higher than these of gutweed protein. The BV of the protein from *Enteromorpha* was 65.19%, so about 65% of the

protein was used by the body for body protein conversion. Similar to other plant proteins, the PDCAAS was not high when compared to the needs of children because the AAS of histidine/lysine was only 0.8. When comparing to adult needs this value was 0.63. It is caused by the AAS of lysine which was only 0.91.

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## CONSUMERS' DRIVERS FOR ACCEPTANCE OF VIETNAMESE COFFEES. APPLICATION OF CATA QUESTION AND PREFERENCE MAPPING

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### ABSTRACT

Identifying the sensory properties that affect consumer preferences for food products is an important feature of product development. Methods to collect sensory profiles relying directly on consumers' perceptions are increasingly employed. Among those, Check-all-that-apply (CATA) questions have seen a widespread use recently. In this study, a consumer test simultaneously collecting information about the consumer background, hedonic ratings and a sensory profile by the CATA technique was reported. The objective of this work was to provide product characterizations and identify drivers of liking as well as specific improvements for individual products.

A total of 88 consumers described the sensory characteristics of the 8 Vietnamese coffees on a CATA questionnaire and evaluated acceptability that the coffees elicited. After Hierarchical Cluster Analysis (HAC) on the liking scores, the data were subjected to Cochran's Q to test for product differences across all attributes. Correspondence Analysis was used to understand how the consumers perceived and described the differences in coffees. Penalty-lift analysis was conducted to explain the differences in hedonic ratings among the coffee samples and to determine the attributes that had significant positive and negative effects.

Three clusters of consumers were identified as a function of different preference patterns. Cluster 1 (n=29) and cluster 2 (n=39) showed the significant different between coffee samples in liking, while cluster 3 (n=19) did not figure out the differences. With regards to drivers of (dis)liking, the results indicated that the attributes *Sweet*, *Vanilla*, *Hazelnut*, *Butter* had positive impact, while *Roasted* and *Astringent* had negative impact on the consumer liking.

**Keywords:** acceptance, check-all-that-apply, coffee, penalty-lift analysis

### 1 INTRODUCTION

With the total consumption of 35000 tons in 2016, coffee is one of the most popular beverages in the Vietnam. Regarding market share, Nestlé has led in Vietnam market with 29% value share,



followed by two domestic companies which account 48% value share [14]. In Vietnam, the drip filters, instead of coffee machines, were used to brew coffees. Consequently, Vietnamese coffees have some specific tastes and aromas which differentiate from other

Coffee is a product that can be drunk on its own or mixed with others (milk, sugar, condensed milk, etc.), making it a typical segmented product for which groups with well differentiated consumption patterns can be identified [28]. In addition to this, sensory properties have been found to be influenced by several factors such as plant varieties, region, processing and brewed method [21]. Several studies have been conducted in many countries using both trained panels and consumers. With trained panels, [8] found 15 sensory attributes in green and roasted coffee beans; [26] developed 16 terms were unique sensory attributes influenced by culture. Also, sensory characteristics of brewed coffee was determined using consumers in [17, 21].

Bitterness is generally considered a negative attribute in food, yet many individuals enjoy a certain amount of bitterness in products such as coffee, beer, or dark chocolate [16]. Understanding sensory characteristics in the process of new product development is of great importance, as failure to obtain correct information about the sensory attributes may lead to fast disappearance of the new products from the marketplace [19].

Check All That Apply (CATA; [1]) questions have recently been used in consumer studies to determine which attributes consumers expect to find in a food product. This method has been applied on a wide range of products such as ice-cream [13]; strawberry [7]; milk dessert [3]. Many studies have shown the similar results as used both consumers and trained panels [11, 13]. For brevity, in CATA, assessors were asked to taste products and select all the words they consider appropriate to describe a product [5]. CATA provides information on which attributes are detectable according to consumers and how that may relate to their overall liking and acceptance. If liking of the products under investigation has been rated along with the CATA question(s), a penalty-lift analysis might be performed, providing an estimate of the average change in liking due to this attribute applying compared to not applying as indicated in the CATA questions [29]. Furthermore, to understand the relationship between consumer and sensory data, preference mapping is a useful method [12]. Recently, some authors [4, 13, 23] have used results from CATA questions for external preference mapping.

In the context, the objectives of this study were (1) to apply CATA questions to characterize the sensory properties of brew prepared roasted coffee, and (2) identify drivers of consumer liking.

## **2 MATERIALS AND METHODS**

### **2.1 Coffee samples**

A total of sixteen samples were collected from four top suppliers (78% value share in Vietnam market). The samples include a range of commercial roasted coffees. All samples were commercially available and had been purchased from retailers in local supermarket.



The samples were prepared according to the following procedure (recommended by suppliers): Step 1-Boil the water using a kettle; Step 2-Place 100g of coffee grounds in drip filter and gently tap it to level the surface of the grounds; Step 3- Pour 150 ml of hot water over the coffee. After giving the coffee 5 minutes to drip, add 200 ml of hot water to drip filter. The confusion was strained through a steel coffee strainer into a beaker.

In the sensory test, 30 ml brewed coffee was presented to the panelist in a 3oz cup labeled by 3-digit random numbers and served at approximately 60°C.

## **2.2 CATA questions**

Eighty-eight consumers were recruited for the test in the south of Vietnam (approximately 50% male, aged between 18 to 45 years). The recruitment was based on the following criteria: drink coffee at least 3-4 days per week, eat at least 2 hours and not use any strong flavor products at least 30 minutes before testing.

Eight products were presented in monadic sequence, following a balanced rotation order. The consumers were asked to taste sample and answer CATA questionnaires by selecting all the terms they considered appropriate to describe each of sample [6, 13, 27]. The consumers were asked to rinse their mouth with un-salted crackers and water between samples [17] .

The CATA questions included a list of 3 colors (Black, Brown red, Brown black), 23 flavors attributes (Cigarette, Roasted, Caramel, Hazelnut, Fish-sauce, Woody, Alcohol, Vanilla, Smoked, Grassy, Drug, Butter, Chocolate, Fruity, Chestnut, Bread, Oxidized, Salty, Bitter, Sweet, Sour, Astringent, Viscous) and 4 aftertastes (Sweet, Astringent, Sour, Bitter).

## **2.3 Data analysis**

Segments of consumers were identified using Hierarchical Clustering Analysis (HAC; Euclidean distance, Ward's method).

Cochran's Q test [10] was carried out on the CATA data in order to identify significant differences between samples for each of the attributes.

Correspondence analysis (CA) [15] was performed to determinate a projection of the data into orthogonal dimensions such that they sequentially represent as much of the variation in the data as possible. To avoid the impart of the rarely selected CATA terms on the final results, the so-called Hellinger distance, instead of Chi-squared distance, was used in CA [18, 24].

Penalty-lift analysis [29] was also performed on consumer responses to determine the effects of the presence and absence of CATA attributes on consumer preferences. In general, liking is averaged across all observations in which the attribute of interest was used to characterize the product, and across those observations for which it was not. Determining the difference between these two mean values provided an estimate of the average change in liking due to the attribute applying compared to not applying in the CATA questions [22].

All data were carried out using R version 3.4.1 [25]

### 3 RESULTS AND DISCUSSION

A total of sixteen commercial samples from commercial coffees were sorted into the groups according to their similarity and dissimilarity. Consequently, eight samples (VN.BL, HL.TT, HL.MO, TN.ST5, PL.RBB, PL.CAR, TN.D1, TN.D2) were chosen for the next analyses.

ANOVA results showed the significant differences in overall liking among samples (Table 1). Tukey's test was used to find means that are significant different from each other.

**Table 1:** Overall liking for eight samples

	VN.BL	HL.TT	HL.MO	TN.ST5	PL.RBB	PL.CAR	TN.D1	TN.D2
<b>Liking</b>	4.23 <sup>ab</sup>	3.41 <sup>b</sup>	3.57 <sup>ab</sup>	4.06 <sup>ab</sup>	3.99 <sup>ab</sup>	4.38 <sup>a</sup>	4.39 <sup>a</sup>	3.97 <sup>ab</sup>

*Different letters indicate statistical differences ( $p < 0.05$ ) among the samples.*

The samples PL.CAR and TN.D1 obtained the highest values for acceptability scores (4.38 and 4.39, respectively). Conversely, the sample HL.TT was the most disliked with the score of 3.41. The rest of the samples did not differ significantly in overall acceptability as compared to the above groups. However, the overall liking scores were low values, less than 5 point (5="neither like or dislike") on a 9-point hedonic scale. In this case, it is not reasonable to conclude the sensory attributes which enhance or suppress the liking scores. For this reason, Hierarchical Cluster Analysis (HCA) was used to classify consumers based on their overall liking scores.

Based on HCA (Euclidean distance, Ward's method), three segments of consumers were initially detected, including Cluster 1 ( $n = 29$ ), Cluster 2 ( $n = 39$ ) and Cluster 3 ( $n=19$ ). In Cluster 1 and Cluster 2, samples were detected significant differences ( $p$ -value = 0.001 and 0.009, respectively) whereas consumers in Cluster 3 did not find any significant differences between samples. However, the scores in Cluster 2 were still below 5, only Cluster 1 was thus used for the further analyses. The means and significant differences between samples for each segment were presented in Table 2.

**Table 2:** Mean overall liking scores for the coffee samples

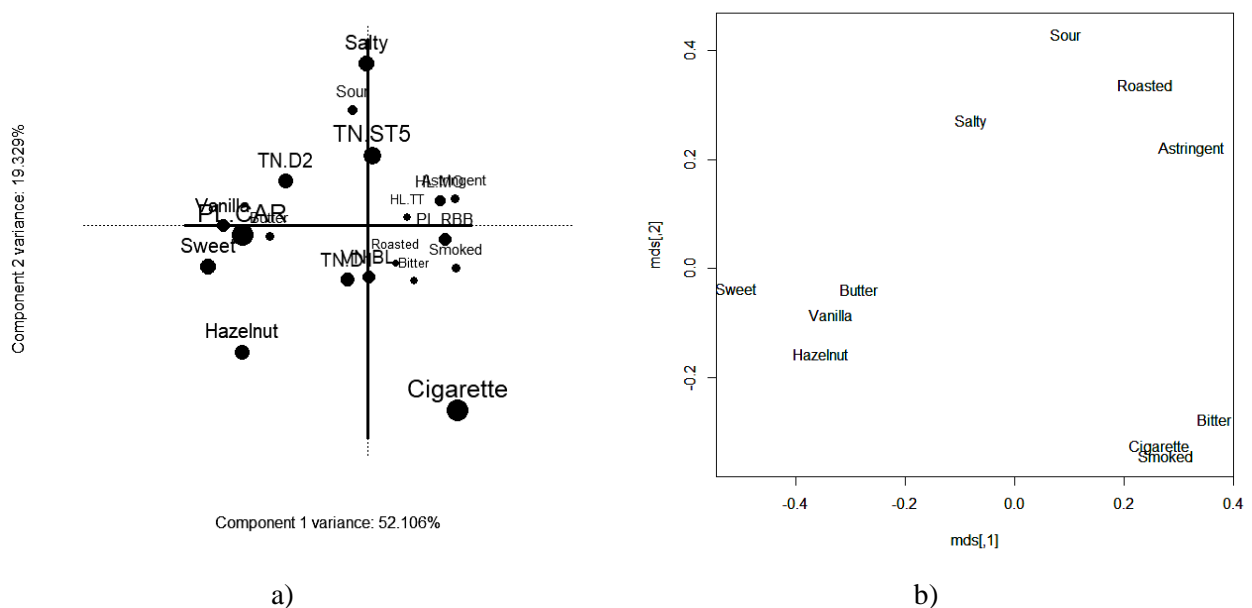
	VN.BL	HL.TT	HL.MO	TN.ST5	PL.RBB	PL.CAR	TN. D1	TN. D2
<b>Clus1</b>	5.59 <sup>ab</sup>	4.76 <sup>b</sup>	4.83 <sup>b</sup>	5.51 <sup>ab</sup>	5.28 <sup>b</sup>	6.76 <sup>a</sup>	5.41 <sup>ab</sup>	5.48 <sup>ab</sup>
<b>Clus2</b>	4.44 <sup>ab</sup>	3.28 <sup>b</sup>	3.36 <sup>b</sup>	3.95 <sup>ab</sup>	3.69 <sup>ab</sup>	3.72 <sup>ab</sup>	4.56 <sup>a</sup>	3.72 <sup>ab</sup>
<b>Clus3</b>	1.74 <sup>a</sup>	1.63 <sup>a</sup>	2.11 <sup>a</sup>	2.05 <sup>a</sup>	2.63 <sup>a</sup>	2.11 <sup>a</sup>	2.47 <sup>a</sup>	2.16 <sup>a</sup>

*Different letters in the same row indicate statistical differences ( $p < 0.05$ ) among the samples.*

For Cluster 1, the ANOVA of acceptance scores showed significant differences among samples. The highest liking values was found for sample PL.CAR (6.76 point on 9-point hedonic scale) whereas samples HL.TT, HL.MO and PL.RBB received the lowest values (4.76, 4.38 and 5.28, respectively). Without considering the values, trends in this cluster did not differ much from the total consumer in term of acceptance ratings except for sample PL.RBB which received low value in liking score.

Cochran's Q test showed that 11 out of 30 sensory attributes were significant differences among samples (test-level = 0.1). These attributes were almost flavors (*Cigarette, Roasted, Hazelnut, Vanilla, Smoked, Butter*), tastes (*Salty, Bitter, Sweet, Sour*) and one trigeminal attribute (*Astringent*). Often, color attributes were considered as necessary for the identification of coffees, the result, however, did indicate attributes *Back, Brown red, Brown* were not evaluated differently from each sample. In addition, it is noting that attribute Viscous, a texture attribute, were also not essential for sample classifications. Compared with other researches [9, 17, 26], Vietnamese consumers recognized a lot of aroma attributes that were just perceived by trained panel.

Correspondence Analysis (CA) was used to visualize the data. In this case, Hellinger's distance has been used. The resulting plot was shown in Fig. 2a.



**Fig. 2:** Representation of sensory attributes and samples based on CA (a) and the distances between attributes based on MDS on the  $\phi$ -coefficient (b)

Fig. 2a depicts the classification of samples based on sensory characterizations. The first two dimensions accounted for 71.4% of total variability in sensory data. Samples PL.CAR and TN.D2 were separated from samples PL.RBB, HL.MO and HL.TT on the first dimension. On the left side of Dim 1, sample PL.CAR was characterized by the attributes *Vanilla, Butter* and *Sweet*, while samples PL. RBB, HL.MO and HL.TT described by *Astringent, Smoked* and located on the

right side of Dim 1. The second dimension differentiated mainly on *Salty*, *Sour* which associated with sample TN.ST5. The size of the circles in Fig. 2a indicated how much the respective product or attribute contributed to the total variance in the data. Interestingly, the attribute *Cigarette*, which highly contributed to the total variance in the data, was not correlated with any specific samples. An attempt to further investigate the relationship between attributes, the  $\phi$ -coefficient for all pairs of attributes was determined based on the full data set [20]. Overall, the association was presented in Fig. 2b. As expected, the attributes *Sweet*, *Butter*, *Vanilla* and *Hazelnut* were found to be high correlation, while the rest attributes seemed to be separated into 2 groups of attributes, *Salty*, *Sour*, *Roasted*, *Astringent*, on one hand, and *Bitter*, *Cigarette*, *Smoked*, on the other hand.

In addition, the external preference mapping was employed to show the sample ideal area with dimensions derived from CA of CATA counts (the plot now shown here). The contour line showed lines in responsive to different percentage of consumers who scored their liking larger than the average liking score provided by the consumer of interest. Sample PL.CAR was located in the area in which the percentage of consumers liked them was 80%. Also, sample TN.D2 was positioned in the area with 60% of consumers liked. On the other hand, samples HL.MO, PL.RBB, HL.TT were located close to areas where the consumers scored their overall liking lower than average liking ranged from 30% to 40%. These results are in agreement with those previously discussed. Noted that the scores here were not very high in 9-point hedonic scale, so it should be careful to conclude that products could be developed with sensory characteristics similar to those of samples in the optimum area.

As shown in Table 2, average overall liking scores ranged between 4.76 and 6.76. Significant difference between samples were found ( $p$ -value = 0.1). In order to examine the impact of different attributes on liking, a penalty-lift analysis was performed. The result indicated that most attributes had positive effect on liking. Not surprisingly, attributes *Vanilla*, *Sweet*, *Hazelnut* and *Butter* were found to be the main drivers of liking whereas attributes *Roasted* and *Astringent* were the main drivers of disliking throughout evaluation. The existence of *Vanilla*, *Sweet*, *Hazelnut* and *Butter* helped to increase overall liking scores up to 2.5 points on the 9-point scale. The attributes seemed to be “motivation” factors, leading to an increase in consumer liking. The result from penalty-lift completely corresponded with the liking scores in Table 2 and Fig. 2a in which PL.CAR was the most liked and characterized by the attributes *Vanilla*, *Butter* and *Sweet*. Similarly, HL.TT, HL.MO and PL.RBB were the most disliked samples and then highly associated with the attributes *Roasted* and *Astringent*.

From the results of penalty-lift analysis, it is noteworthy that the attribute *Cigarette* or other related attributes (*Smoked*, *Bitter*) were not the drivers for disliking at all. In other words, an attribute is essential for sample configurations, it is not sure that the attribute was the driver for (dis)liking. This result is in agreement with the finding as highlighted by [2].

## **4 CONCLUSIONS**

Determining the drivers for consumer liking of a particular product, Vietnamese coffee in this case, is a complex action that depends on a wide range of factors including both intrinsic and extrinsic factors. The use of CATA question could be an interesting and simple methodology to get an insight on consumer perception of a food product. The present study identified the sensory profile of Vietnamese coffees, and indicated some drivers for (dis)liking, leading to obtain actionable directions for food product development.

In this study, eighty-eight consumers were recruited for consumer test, it seems to be large enough. However, only Cluster 1 (n=29) indicated the differences in liking and thereby it should apply to larger samples size in further studies. From that, the conclusions would be clearer.

## **5 ACKNOWLEDGMENTS**

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## **ADDITION OF VEGETABLES IN THE EXTRUSION OF RICE PUFFED SNACKS TO INCREASE VEGETABLES CONSUMPTION IN CHILDREN**

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### **ABSTRACT**

Children's low consumption of fruit and vegetables have been a concern in Indonesia. Even until recently, Indonesia still cannot fulfil the recommended fruit and vegetables consumption per capita. A survey done in four elementary schools in Central Java revealed that approximately 45,6% of the children do not consume vegetables daily and approximately 1,6% students has never eaten vegetables. This may lead to a lot of health problems. To overcome this problem, innovations are needed. One of the innovations that can be done is the addition of vegetables into extruded snack products which are children's favorite snacks. Extruded snacks from rice and vegetables were made with different vegetables concentrations (0%, 5%, 10%, 15% and 20%). Vegetables used were carrot and cassava leaves which is a rich source of beta-carotenes. Based on the results, as the concentration of vegetables increased, the physical quality, such as texture and color of the extrudates decreased. Based on the physical characteristics and the beta-carotene content of the extrudates, it can be concluded that the optimum concentration for carrot and cassava leaves addition was 15% and 10%, respectively. With the addition of 15% carrot and 10% cassava leaves, the beta-carotene increased 13 times and 22 times, respectively, without significantly altered the physical characteristics of the extrudates. The amount of beta-carotene in 100 grams of 15% carrot extrudates fulfil 18% of vitamin A RDI and 100 grams of 10% cassava-leaves extrudates fulfil 29% of vitamin A RDI.

**Keywords:** vegetable, rice, extrusion, children, snack

### **1 INTRODUCTION**

The low consumption level of fruits and vegetables especially in children has always been a concern. Low consumption of vegetables has long been associated with various health problems such as cardiovascular diseases [1], lung problem [2] and various other non-communicable diseases [3]. Therefore WHO and FAO recommends to consume fruits and vegetables more than 400 g/day [4].



Attempts to increase the consumption level had been done through various approach such as education through school, parents, peer and media [3], [5]. Some approach may be more effective than the others. In order to asses children's vegetable consumption level and factors which may affect the vegetables consumption, specifically in Central Java, Indonesia, a survey was done to four elementary schools in Central Java as a preliminary study.

In order to increase children vegetable consumption, products development may also be done to the snacks that children usually consume, for example extrudates. Usually extrudates is made from corn with addition of flavoring. Vegetable flakes can be added to the extrudates in order to increase the intake of vegetables in children.

This research aims to study the effects of vegetables flakes addition to the physicochemical characteristics of the extrudates. Hopefully with the addition of vegetables to puffed snacks that children enjoy may increase children inclination to consume vegetables.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

The rice used in this study is local white rice variety C4 obtained in the local market. The chantenay carrot and the cassava leaves were both obtained from the local market.

### **2.2 Preliminary Study**

A survey was done to study children's vegetable consumption. The survey is done at four elementary school in central Java, two school represented middle-high-income family and two school represented low-middle-income family. All the four school is located in Central Java Province but in different cities. Two questionnaires were used to obtain information about the vegetable consumption frequency, types of vegetable consumed and factors that may affect children's consumption. The questionnaires were directed to the children and their mother. The total respondent were 246 pairs of children and mothers.

### **2.3 Extrusion process**

The extrusion process was done using a single screw extruder with white rice only. The addition of vegetables substituted the weight of rice for 5%, 10%, 15%, and 20%. After extrusion process, the extrudates were dried in a cabinet drier in 60°C for 4 hours.

### **2.4 Physical Characteristic Analysis**

#### *2.4.1 Expansion Ratio*

Radial expansion ratio was measured as the ratio (in %) between extrudates diameter and the die diameter. Longitudinal expansion ratio was measured as the length of each extrudates that was cut using rotating knives with a constant speed.

#### 2.4.2 Specific Density

Specific density was measured as the ratio between the mass of one piece of extrudates and its volume. The volume of the extrudates was measured by seed displacement method.

#### 2.4.3 Hardness and Crispness

Hardness and crispness of the extrudates were measured using Lloyd Texture Analyzer with cylinder probe, test speed 1 mm/s, depression limit 8 mm. Hardness was expressed as the maximum force and crispness was expressed as the number of peaks in the force-deformation graph.

### 2.5 Beta-carotene Content Analysis

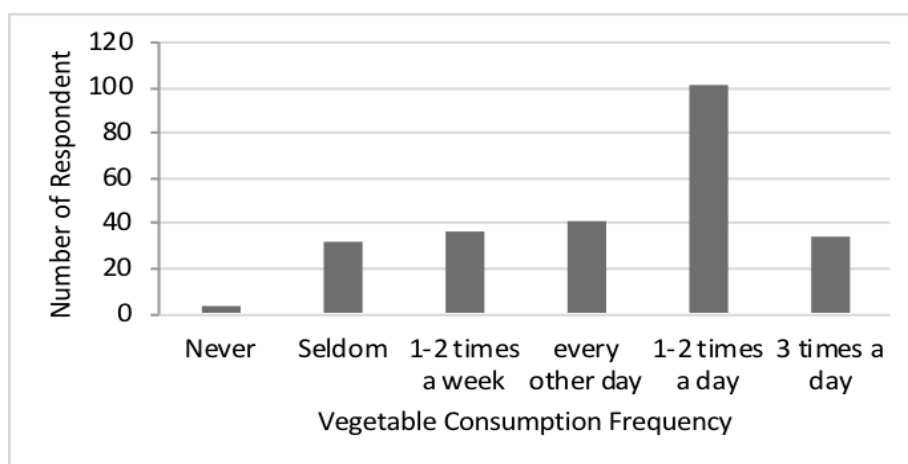
Beta-carotene from 5 gram of grounded extrudates was extracted with acetone, hexane and  $\text{MgCO}_3$  for 18 hours. The extract was then filtered using chromatography column consist of filter cloth, celite powder,  $\text{MgO}$  powder and  $\text{Na}_2\text{SO}_4$ . The absorbance of the filtrate was then measured using spectrophotometer at 436 nm. The concentration of beta carotene was calculated using standard curves and was expressed in mg/100 g.

### 2.6 Data analysis

The data obtained from the extrudates physicochemical analysis were then statistically analyzed using One Way Anova with Duncan test.

## 3 RESULTS AND DISCUSSION

### 3.1 Level of Vegetable Consumptions in Children



**Figure 1:** Frequency of Vegetable Consumption in Elementary School Children

From Figure 1, it can be concluded that 1,6% children never even consumed vegetables. As much as 45,6% of the children does not consume vegetable daily which obviously does not meet the requirement of daily fruits and vegetables consumption by the WHO/FAO. Therefore, a strategy is needed to improve children's vegetable consumption in Central Java, Indonesia.

From the survey, significant factors that affect children's vegetable consumption were mother's behavior, i.e. regularly buying vegetables (cor. coef. 0.642), providing children with lunchbox (cor. coef. 0.238), and eating meals together with their children (cor. coef. 0.749). There were also weak significant correlation (cor. coef. 0.283) between mother's perception and children vegetable consumption. Mothers who understand the significance of vegetables to health tend to have children with high vegetable intakes.

The vegetables that are the most familiar to children were spinach and carrots. Meanwhile cassava leaves was the least familiar to children. Carrots and cassava leaves were then selected to be added to the extrudates to increase its nutritional value.

### 3.2 Physical Characteristics of Rice and Vegetable Extrudates

**Table 1:** The Expansion Ratios and Density of Rice and Vegetables Extrudates

Formulation	Radial Expansion (%)	Longitudinal Expansion (cm)	Specific Density (g/cm <sup>3</sup> )
Rice (control)	276,556±17,003	3,331±0,313	0,078±0,008
Rice +5% CL	241,200±17,413	3,756±0,361	0,084±0,005
Rice +10% CL	227,619±22,859	3,998±0,296	0,092±0,008
Rice +15% CL	196,056±16,219	4,084±0,187	0,094±0,009
Rice +20% CL	188,933±11,800	4,479±0,415	0,126±0,014
Rice +5% CP	246,567±17,306	3,210±0,221	0,084±0,008
Rice +10% CP	229,233±19,552	3,864±0,366	0,086±0,007
Rice +15% CP	222,000±22,182	3,880±0,282	0,103±0,010
Rice +20% CP	204,000±20,219	3,914±0,209	0,112±0,008

*Note: CL: cassava leaves; CP: carrot*

The addition of vegetable powder, either carrot powder or cassava leaves, caused the radial expansion during the extrusion to decrease. In the melt, the insoluble fiber from the vegetable flakes tend to exist as filler in the starch and to align parallel to the flow direction of the melt [6], [7]. Thus, decreasing radial expansion as the concentration of the vegetables increased were observed. However, the lack of radial expansion were compensated by the increase in the longitudinal expansion. The more vegetables were added, the higher the longitudinal expansion were during extrusion. This also happened in previous studies where fibrous materials were added into extrusion process [8], [9]. This is due to the fiber in the vegetables which allign with the matrix of the starch to limit the radial expansion, then part of the excessive energy were used

to expand longitudinally. However, overall expansion were still decreasing with higher concentration of vegetables added and this caused the specific density of the extrudates to increase which indicated a more compact product with lower porosity.

Due to the increasing density and decreasing radial expansion, increasing hardness were also observed in the extrudates. Decreasing radial expansion also means that the number of pores decreased and the air cell wall thickness increased which caused the reduction in crispness. However, compared to a commercial product which is widely consumed by children with hardness  $2210,17 \pm 484,74$  and crispness  $8.88 \pm 2,25$ , the addition of cassava leaves up to 20% and carrot up to 15% still gave an acceptable level of hardness. However, the addition of more than 10% cassava leaves and more than 15% carrot may decrease the crispness into an unacceptable level.

In terms of color, the addition of carrot and cassava leaves gave their natural color to the extrudates. The extrudates with cassava leaves showed a pronounced green color while the extrudates with carrot did not have a significant change in color. The concentration of the vegetables added did not significantly affect the color of the extrudates up to the concentration of 10%.

**Table 2:** The Texture and Color of Rice and Vegetables Extrudates

Formulation	Hardness (gf)	Crispness	L*	a*	b*
Rice (control)	1423,44±100,02	7,90±0,74	86,61±4,21	-0,24±0,02	11,99±0,45
Rice + 5% CL	1676,93±155,07	7,30±1,42	75,52±3,64	-2,81±0,12	21,42±0,65
Rice + 10% CL	2005,30±105,16	7,10±0,74	71,42±1,11	-2,86±0,13	21,31±0,82
Rice + 15% CL	2063,94±221,42	6,50±0,85	68,53±2,26	-3,08±0,11	21,73±0,93
Rice +20% CL	2185,10±202,70	4,90±0,88	63,82±3,03	-3,43±0,09	21,75±1,01
Rice +5% CP	1502,34±223,49	7,82±1,17	86,73±0,80	-0,25±0,02	19,58±1,40
Rice + 10% CP	2043,44±270,27	7,16±1,43	86,01±0,31	-0,27±0,03	24,22±2,56
Rice + 15% CP	2272,58±380,49	7,05±1,62	83,08±1,26	-0,31±0,03	28,75±1,00
Rice + 20% CP	2443,03±383,87	6,40±1,51	83,09±1,44	-0,35±0,03	31,23±1,38

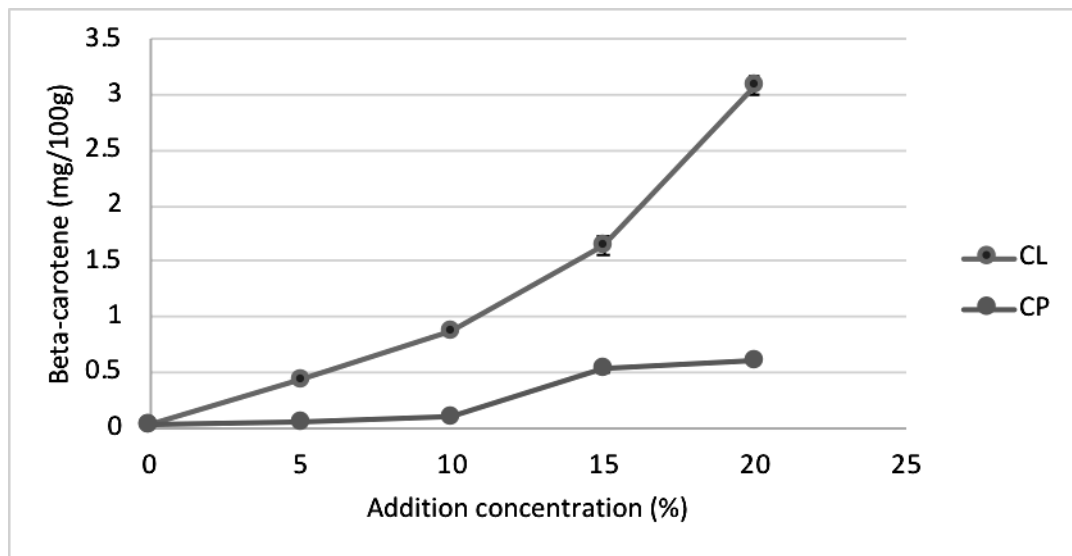
Note: CL: cassava leaves; CP: carrot

### 3.2 Beta-carotene Content of Rice and Vegetable Extrudates

The beta-carotene content of the extrudates can be seen in Figure 2 below. As expected, with the increasing concentrations of the vegetables, the beta-carotene content were increased. Extrudates with cassava leaves had a significantly higher beta-carotene content than extrudates with carrot. Compared to the rice extrudates without vegetables addition, the addition of 15% carrot and 10%

cassava leaves increased the beta-carotene 13 times and 22 times, respectively, without significantly altered the physical characteristics of the extrudates.

The amount of beta-carotene in 100 grams of 15% carrot extrudates fulfils 18% of vitamin A RDI and 100 grams of 10% cassava-leaves extrudates fulfils 29% of vitamin A RDI. One commercially available puffed-snack which contain vitamin A has 128 IU of the vitamin. Thus, with the assumption that 1 IU equals to 0.6 µg beta-carotene [10], extrudates with 10% cassava leaves has 11 times more Vitamin A. Extrudates with 15% carrot has 7 times more vitamin A than the commercial products.



**Figure 2:** Beta-carotene content in Extrudates with different concentrations of cassava leaves and carrot addition

## 4 CONCLUSIONS AND FUTURE STUDY

The addition of cassava leaves and carrot powder increases the beta-caroten level of extrudates significantly. This opens an opportunity to improve children's intake of vegetables. However, the vegetable addition may also lead to the degradation of extrudates' physical quality. Conventional extrusion caused significant changes in the physical quality of the extrudates. In order to improve the physical quality, supercritical-fluid extrusion may be used.

Supercritical fluid extrusion uses supercritical CO<sub>2</sub> as the expansion driving force instead of steam during extrusion. This results in a better expansion control and minimum nutrition degradation [11]. Research has been done to extruded milk protein [12] and also with addition of fibrous materials such as fruit pomaces [13].

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## PRODUCT DEVELOPMENT OF PORK-RICE SNACK USING THREE THAI RICE WITH CORN FLOUR AND POTATO STARCH

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### ABSTRACT

Rice-based snack incorporating with sweet pork was developed to utilize Thai rice and improve snack texture. Five ratios of pork: rice (30:70, 40:60, 50:50, 60:40 and 70:30) were prepared and evaluated by 9-point-hedonic preference method with 30-untrained panelists. Ratio of pork: rice 30:70 sample was chosen as basic formula due to the highest score of overall liking ( $6.00 \pm 2.02$ ) and higher score in some attributes. Factorial design ( $3 \times 2 \times 2$ ) were prepared using 3 Thai varieties of rice (Jasmine-Rice (JS), Brown-Rice (BR) or Rice-Berry Rice (RB)), 2 types of starch (Corn Starch (CS) or Potato Starch (PS)) and 2 starch content (5 or 10% of pork-rice snack). Samples were evaluated by 9-point-hedonic preference method with 30-untrained panelists and texture (hardness) was also analyzed by texture analyzer. Varieties of rice might affect color attributes. 2 out of 12 samples, JR-5% CS and BR-5% CS were gained high score in color, crunchy, hardness, chewiness and overall-liking attributes and hardness (measured by texture analyzer). BR-5% CS was chosen to carry out consumer survey with 100 consumers. 72% of consumers accepted the pork-rice snack and rated overall liking score about  $6.5 \pm 2.3$ .

**Keywords:** Pork-Rice Snack, Jasmine Rice, Brown Rice, Rice-Berry Rice, Corn Starch, Potato Starch, Pork

### 1 INTRODUCTION

There are some kind of rice snack used rice or glutinous rice as a main raw material such as Senbei (Japan Rice cracker), Yukwa (Korea traditional puffed rice cracker), Thai rice cracker etc. Rice was milled, added with water and seasoning, kneaded, shaped, steamed, dried and baked and puffed by frying in cooking oil (Shin, *et. al.*, 1990; Charunuch *et. al.*, 2008; Keeratipibul *et. al.*, 2008 and Noomhorm *et. al.*, 1997). Crispness and crunchiness are the necessary characteristics of rice cracker. In term of nutrients, rice is high in carbohydrate but low in protein. On the other hand, there are many meat crackers that use meat such as pork, beef, chicken mixing with salt, sugar, spices, with/without flour, marinating, shaping, battering, cooking, frying or drying such as nugget, fry chicken, Jerky (Ray *et.al*, 1996; Konieczny *et. al*, 2006; Yang *et. al.*, 2009; Ngadi *et. al.*, 2009). Meat snack is high in protein but its texture is too sticky and therefore cannot be kept



at room temperature. The combination of rice cracker and meat snack might satisfy customer need of carbohydrate, protein and other micronutrients. Convenience, lighter weight, cheaper price, simple conditions for storage, crispy or crunchy texture are some added properties of product. In addition, the flavor of pork and rice also have been mentioned in an account of final product.

The purpose of this study was to develop a snack food product incorporating with nutritional diets from varieties of rice Brown rice, Jasmine rice or Riceberry as a main source of carbohydrate and other micronutrients and high protein source from pork.

## **2 MATERIALS AND METHODS**

### **2.1 Product Development of Pork-Rice Snack**

#### *2.1.1 Ratio of pork and rice*

Pork-rice snack was separated into two major parts including (i) ground pork portion and (ii) cooked-rice paste. The first part, pork shoulder was bought from supermarket in Bangkok and removed subcutaneous fat and connective tissue. Pork was grinded by Grinder (Savioli® TRC22BE). Ground pork was mixed with 12% sugar, 2.76% soy sauce, 2.76% oyster sauce, 2.76% sesame oil, 1.97% rice wine, 1.66% fish sauce, 0.18% black soy sauce, 0.36% ground white paper and 0.1% five-spices powder containing anise, cinnamon, clove, fennel and watchou (modified from Chen, Liu and Chen, 2002). Ground-pork portion was kept under 4°C for 24 hrs. The second portion, cooked-rice paste was prepared by blending 2-parts of milled rice and 1-part of milled glutinous rice in blender (Buno®, BUO-12KP61). Then it was cooked about 10 min to form a pre-gel paste and mixed with the first part or the ground-pork portion. After that the mixed paste was spread on a tray and was dried by hot-air dryer at 60°C for 4 hrs. The dried sheet of pork-rice snack was cooled and cut into small pieces with approximate of 35x35mm and kept in vacuum plastic box.

The proper formula of pork-rice snack was conducted by mixing different percentage of ground pork to cooked-rice paste as 30:70, 40:60, 50:50, 60:40 and 70:30, respectively. Ground pork and cooked-rice paste were weight separately and mixed well. All samples were dried by hot-air dryer, cooled, cut and fried before serving to panelists. Samples were evaluated by sensory panel for color, pork flavor, rice flavor, crispiness, hardness, toughness, oiliness and overall liking. The experiment was performed in replicated.

#### *2.1.2 Effect of different types of rice and effect of starch and its percentage*

The suitable formula judged by panelists from 2.1.1 was used to study the effect of rice, starch and percentage of starch to the pork-rice snack. A 3×2×2 factorial design was employed to investigate the influence of 3 different types of rice (Jasmine rice, Brown rice and Riceberry), 2 different types of starch (corn and potato starch) and 2 different percentage of each starch (5 and

10%) on the physical property (hardness) and sensory properties (color, overall flavor, crunchiness, hardness, chewiness and overall liking) of pork-rice snack. The experiment was performed in replicated.

### *2.2.3 Consumer survey*

A consumer group (n=100) including undergraduate, graduate and staffs of Assumption University, Bangkok, Thailand, were invited as taste panelists to evaluate the product. The questionnaire was divided into three parts (i) customer's behavior about snack, (ii) customer's attitude and needs toward the development products and (iii) demographic data. Consumer choices were scored on a five-point scales from 'not important' to 'very important'. Sensory analysis was carried out by rating product attributes: appearance, color, sweetness, hardness, crispiness, and overall acceptance of the final sample (using 9-point hedonic scales, where 1 = extremely dislike and 9 = extremely like). The questionnaire was the tool for analyzing consumption patterns, frequency of purchasing and demographic data.

## **2.2 Analytical methods**

### *2.2.1 Sensory Analysis*

*Sample preparation:* 35×35mm square of pork-rice snack was fried in deep fryer at 177°C and placed on paper to absorb excess cooking oil and kept in vacuum plastic box. Samples was kept overnight before serving at room temperature.

*Sensory analysis for 2.1.1:* Samples were evaluated by 30-untrained panelists in Bangkok. Random hedonic test was carried out using 9-point scales (1 = extremely dislike to 9 = extremely like) in which the panels evaluated different attributes: color, pork flavor, rice flavor, crispness, hardness, toughness, oiliness and overall liking.

*Sensory analysis for 2.1.2:* The samples were offered in small plastic white cups coded with random three-digit numbers. Each panelist evaluated twelve samples in a balanced sequential order. The optimal rice (Jasmine rice, Brown rice and Riceberry), starch (corn starch and potato starch) and ratio of starch (5 and 10% for each starch) in pork-rice snack were investigated by comparing sensory quality of samples. Sensory evaluation was repeated on two different days. Due to the experimental design that had 12 samples so the evaluation by sensory test was divided into two sessions. First six samples were evaluated in the first week and another six samples were evaluated in the following week by the same group of panelists. The preference test was used in screening the most preferable type of rice, starch and percentage of starch for pork-rice snack development. The 9-point hedonic scale preference test was used to select the suitable type of rice, starch and ratio of starch and its combination was accepted and used for further studies.

### 2.2.2 Texture analysis

The hardness was determined by the TAXT plus Texture analyzer (Stable Micro System®) with Crip Fracture Rig (PHDP/CFS) and heavy-duty platform (HDP/90) (50-kg. load cell, pre-test speed, 1.0mm/s, test speed 1.0mm/s., post-test speed 10mm/s and distance 3mm). Each sample was measured with seven duplications.

### 2.2.3 Statistical analysis

The experiment data were analyzed by a statistical analysis system, and subject to analysis of variables (ANOVA) analysis and Duncan's multiple range test for significance in comparison of mean ( $P < 0.05$ ).

## 3 RESULTS AND DISCUSSION

### 3.1 Ratio of rice and pork

Table 1 showed the average scores from 9-point hedonic preference test in different ratio of pork and rice. There were significant difference in pork flavor, crispiness, hardness, toughness and overall liking ( $P < 0.05$ ). There were four attributes crispiness, hardness, toughness and overall liking which had higher scores given for ratio 30:70 and 40:60 samples. The hardness, toughness and overall liking of sample with ratio of 50:50 were quite remarkable whereas the ratio 60:40 and 70:30 samples obtained lower scores for these properties.

The color of the sample with ratio of 40:60 had significantly highest score ( $6.7 \pm 1.3$ ). In contrast, the ratio of 70:30 sample obtained lowest score ( $6.2 \pm 1.1$ ). There was no significant difference between samples in liking score on 9-point hedonic preference test of color, rice flavor and oiliness ( $P < 0.05$ ). Browning color in fried food products was the result of Maillard reaction which is a non-enzymatic browning reaction between amino acids and reducing sugars (Baik and Mittal, 2003; Moyana *et al.*, 2002). The addition of pork represented the amount of amino acid for the formula. Thus, the Maillard reaction easily occurred.

The higher percentage of pork led to the increase of pork-flavor score which was higher in sample with ratio 70:30 comparing with other samples (ratio of 30:70 and 60:40). In contrast, the higher pork-portion reduced snack-preference scores in crispness, hardness, toughness and overall liking. Preference scores of samples with ratios from 30:70 to 50:50 were higher than these of ratios from 60:40 to 70:30. The pork flavor showed that the panels preferred the rice-based snack containing high percentage of pork. Similar to previous researches, processed meats with higher levels of sweetness were preferred (Chen *et al.*, 2002). The higher percentage of pork enhanced the sweetness in the rice-based snack due to the sugar which had been mixed with meat as one of ingredients. Fat content and texture are important quality parameters of fried product. The score of texture attributes included hardness and toughness showed that the panelists preferred pork-rice snack made with ratio from 30:70 to 50:50. However, the crispness decreased

from ratio of 30:70 to 60:40 samples. Crispness and hardness were influenced by the carbohydrate of rice, the starch in rice was denatured easily by thermal treatment such as pre-gelatinization, drying and frying so the starch granule rearranged and improved the hardness and crispiness. The frying process modified the physical, chemical and sensory characteristics of the food (Moyano *et. al.*, 2002). The result was similar to the literature reported by Na-Nakorn (2009). The author showed that the higher degree of disintegration of starch granule during pre-gelatinization was found in low-to-medium amylose content starch comparing to those in high amylose content starch. Another study showed that increasing of starch could improve the hardness (Sirichokworakit *et. al.*, 2016). The results from study showed that the liking score of toughness in different samples with different ratio of pork and rice varied from  $4.6 \pm 2.0$  to  $5.3 \pm 1.9$ . Toughness refers to the brittleness or force required to break the snack on the first bite (Quintero-Fuentes *et. al.*, 1999). The myofibrillar protein structure in meat was modified or denatured by the heat-drying and heat-frying treatment and low percentage of pork could improve the toughness (assessed by a taste panel). The result agreed with the protein denaturation at high temperature in Chinese-style pork jerky (Chen *et. al.*, 2002). The snack sample with ratio of 30:70 of pork: rice was rated with the highest overall liking scores. Panelists liked the samples with high percentage of rice, and texture of product were better. The obtained results are similar to the report of Quintero-Fuentes *et. al.*, (1999). The research mentioned that light crunchy on the first bite are more preferable. In conclusion, sample with ratio of 30:70 was chosen as the basic formula of pork-rice snack due to high score of crispness, hardness, toughness and overall liking and economic reasons of product.

**Table 1:** The average score from 9-point hedonic scale preference test of pork-rice snack using different percentage of pork to rice (30:60, 40:60, 50:50, 60:40 and 70:30)

Pork: Rice	Color	Pork flavor	Rice flavor	Crispness	Hardness	Toughness	Oiliness	Overall liking
30:70	$6.5 \pm 1.3$	$5.8 \pm 1.6^b$	$5.4 \pm 1.7$	$6.2 \pm 2.0^a$	$5.4 \pm 1.9^a$	$5.3 \pm 1.9^a$	$6.3 \pm 1.9$	$6.0 \pm 2.0^a$
40:60	$6.7 \pm 1.3$	$5.8 \pm 1.7^b$	$5.5 \pm 1.7$	$5.7 \pm 2.2^{ab}$	$5.0 \pm 2.0^{ab}$	$5.0 \pm 2.0^{ab}$	$6.2 \pm 1.8$	$5.8 \pm 1.9^{ab}$
50:50	$6.4 \pm 1.4$	$5.8 \pm 1.8^b$	$5.7 \pm 1.9$	$5.5 \pm 1.8^b$	$5.0 \pm 2.1^{ab}$	$4.8 \pm 1.8^{ab}$	$6.1 \pm 1.8$	$5.7 \pm 1.9^{ab}$
60:40	$6.3 \pm 0.9$	$5.9 \pm 1.8^b$	$5.4 \pm 1.5$	$4.4 \pm 1.7^c$	$4.5 \pm 1.8^b$	$4.6 \pm 2.0^b$	$6.1 \pm 1.8$	$5.1 \pm 1.8^c$
70:30	$6.2 \pm 1.2$	$6.5 \pm 1.5^a$	$5.8 \pm 1.3$	$4.6 \pm 2.0^c$	$4.5 \pm 2.1^b$	$4.6 \pm 2.0^b$	$6.2 \pm 1.9$	$5.4 \pm 1.9^{bc}$

Means ( $\pm$  Standard deviation) within a column followed by different letters are significantly different ( $P < 0.05$ )

### 3.2 The different types of rice, starch and percentage of starch

Table 2 presented the different variety of rice including (Jasmine rice (JR), Brown rice (BR) and Riceberry (RB)), different type of starches (corn starch (CS) and potato starch (PS)) and different percentage of each starch (5 and 10%) in pork-rice snack. There were significant difference in

color, crispness, hardness, chewiness and overall liking ( $P < 0.05$ ). Samples made from Riceberry obtained lowest color score for all concentration of corn and potato starch ( $4.1 \pm 1.9$  to  $5.9 \pm 2.1$ ) because Riceberry contains anthocyanin (purple-violet pigment), a major phytochemical (Leardkamolkarn *et. al.*, 2011) which was well soluble in water (Abdel-Aal *et. al.*, 2006) and sensitive to thermal treatment. The pigment was converted into darker color and this color affected the sensory panel and might lead to the lower scores in overall liking. Brown rice contains rice bran layer which expressed as brown color, the brown color also increased the intensity of rice-based snack color. Jasmine rice is a white grain which bran layer are removed. The intensity of the rice-based snack made from Jasmine rice was lighter in color than that of other two variety of rice. The addition of different types of starch and percentage of starch made from Jasmine rice and Brown rice did not affect the color preference. However, the color was significant difference due to the pigment containing in Riceberry. Color score of RB-5% CS decreased from  $5.9 \pm 2.2$  to  $4.1 \pm 1.9$  in RB-10% CS. In addition, Maillard reaction resulted in brown color of snack and enhanced high intensity of color in samples made from Riceberry and Brown rice (Maneerote *et. al.*, 2009). For the effect of starch to color, it was found that 5% to 10% corn starch (w/w) gave higher score for the Jasmine rice and Brown rice snack and lower score for Riceberry snack. JR-10% PS and BR-5% CS samples gave higher score of color. Corn starch would impart the lighter color to the snack as the corn starch made from corn had a high intensity of yellow color.

There was no significant difference in overall flavor among 12 samples ( $P < 0.05$ ). Varieties of rice, starch and percentage of them might not affect overall flavor. The overall flavor was rated from  $5.6 \pm 1.7$  to  $6.4 \pm 1.7$  or neither-like-or-dislike to slightly-like. However, samples containing corn starch had slightly higher score comparing to potato starch in overall flavor. Corn starch affected the overall flavor of pork-rice snack so liking scores increased in some samples. Starches from different cereals were used as functional ingredients to achieve various textural attributes in bake and fried snacks. The crunchiness of snack had high score in the samples made from Jasmine rice and Brown rice containing 5%CS and 10%PS whereas all of the samples contained 5% to 10%CS and 5% to 10%PS made from Riceberry got high score as well. 5% starch (w/w) gave a higher score than 10% starch (w/w) in both corn and potato starch except samples with BR-5%PS or BR-10%PS. The results agreed with the report of Huang (1995). High-amylose of corn cultivars can significantly affect fried chip quality and increase crunchiness.

The satisfaction of panelists in texture of snack (hardness and chewiness) were statistically significant. Both 5 and 10% of potato starch were added into Jasmine rice (JR-5%PS and JR-10%PS) and both 5 and 10% (w/w) were added into Brown rice (BR-5%CS and BR-10%CS). Samples were rated higher score in hardness and chewiness as same as JS-5%CS and BR-10%PS samples. Moreover, the samples made from Riceberry with both corn and potato starch with different concentration (5 and 10% (w/w)) also obtained high scores for hardness and chewiness. All samples contained 5% to 10%CS were consistently rated highest for hardness and chewiness

attributes by panelists (except JR-10%CS) or 5% to 10%PS (except BR-5%PS) in all variety of rice. The liking scores on preference of hardness were ranged from  $5.1 \pm 1.9$  to  $6.40 \pm 1.6$  whereas the liking score on preference of chewiness were ranged from  $5.1 \pm 1.7$  to  $6.3 \pm 1.8$ . For the attribute of overall liking of pork-rice snack, it was found that 5%CS and 10%PS of Brown rice and Riceberry, 5%CS of Jasmine Rice and Riceberry, and 10%PS of Jasmine rice and Brown rice gave high scores.

**Table 2:** Average score of 9-point hedonic preference test of snack made from different rice, starch and starch percentage [Rice (Jasmin rice, Brown rice and Riceberry); Starch (Corn starch and potato starch) and starch percentage (5% and 10%)]

Rice	Starch	%	Color	Overall Flavor	Crunchy	Hardness	Chewiness	Over Liking
Jasmine Rice	Corn Starch	5	$6.7 \pm 1.3^{ab}$	$6.2 \pm 1.5$	$6.7 \pm 1.5^a$	$6.4 \pm 1.9^a$	$6.3 \pm 1.8^a$	$6.4 \pm 1.8^{ab}$
		10	$6.7 \pm 1.5^{ab}$	$6.0 \pm 1.7$	$5.4 \pm 2.1^d$	$5.1 \pm 1.9^b$	$5.1 \pm 1.7^b$	$5.6 \pm 1.8^{bc}$
	Potato Starch	5	$6.1 \pm 1.6^{ab}$	$5.6 \pm 1.9$	$6.1 \pm 1.7^{abcd}$	$5.6 \pm 2.0^{ab}$	$5.7 \pm 1.8^{ab}$	$5.6 \pm 1.8^{bc}$
		10	$7.0 \pm 1.0^a$	$5.9 \pm 1.7$	$5.7 \pm 1.5^{bcd}$	$5.7 \pm 1.5^{ab}$	$5.6 \pm 1.7^{ab}$	$5.9 \pm 1.5^{abc}$
Brown Rice	Corn Starch	5	$7.0 \pm 1.1^a$	$6.4 \pm 1.4$	$6.6 \pm 1.6^{ab}$	$6.1 \pm 1.5^a$	$6.1 \pm 1.6^a$	$6.6 \pm 1.6^a$
		10	$6.5 \pm 2.2^{ab}$	$6.4 \pm 1.8$	$5.7 \pm 1.9^{bcd}$	$5.8 \pm 1.8^{ab}$	$5.9 \pm 1.7^{ab}$	$6.1 \pm 1.6^{abc}$
	Potato Starch	5	$6.7 \pm 1.4^{ab}$	$5.6 \pm 1.7$	$5.6 \pm 2.0^{cd}$	$5.2 \pm 1.8^b$	$5.1 \pm 1.8^b$	$5.5 \pm 1.9^c$
		10	$6.5 \pm 2.2^{ab}$	$6.4 \pm 1.7$	$6.0 \pm 2.0^{abcd}$	$5.7 \pm 1.9^{ab}$	$6.0 \pm 1.5^{ab}$	$6.2 \pm 1.6^{abc}$
Riceberry	Corn Starch	5	$5.9 \pm 2.2^b$	$6.3 \pm 1.2$	$6.8 \pm 1.3^a$	$6.4 \pm 1.6^a$	$6.2 \pm 1.7^a$	$6.4 \pm 1.4^{ab}$
		10	$4.1 \pm 1.9^c$	$6.0 \pm 1.7$	$6.0 \pm 1.8^{abcd}$	$5.8 \pm 1.8^{ab}$	$5.4 \pm 1.7^{ab}$	$5.7 \pm 1.6^{bc}$
	Potato Starch	5	$4.7 \pm 2.0^c$	$6.0 \pm 1.8$	$6.4 \pm 1.5^{abc}$	$6.0 \pm 1.6^{ab}$	$5.6 \pm 1.8^{ab}$	$5.7 \pm 1.5^{abc}$
		10	$5.0 \pm 2.1^c$	$5.9 \pm 1.6$	$6.1 \pm 1.7^{abcd}$	$5.7 \pm 1.9^{ab}$	$5.5 \pm 1.9^{ab}$	$5.7 \pm 1.7^{abc}$

Means ( $\pm$  Standard deviation) within a column followed by different letters are significantly different ( $P < 0.05$ )

It is also found that snack samples made from corn starch obtained higher score for overall liking than that of samples made from potato starch. Samples prepared with Brown rice and 5% corn starch (w/w) gave highest overall liking score, which could be considered as the optimum formula for an acceptable product. This may be related to the excessive or high percentage of starch and low percentage of rice that was responsible for lower texture values (Dogan, Sahin, and Sumnu, 2005; Fiszman and Salvador, 2003) and affected the overall liking. Beside, samples JR-5%CS, BR-5%CS, BR-10%PS, RB-5%CS, BR-5% PS and BR-10% PS also gained high



preference score in all attributes (crunchy, hardness, chewiness and overall liking) except color attributes.

Table 3 showed the p-value (level of significance) of each ingredient and its interaction ( $P < 0.05$ ). Results showed that different types of rice affected the color. The liking score for color preference was significant difference among the samples made from different varieties of rice. Type of starch affected overall flavor and overall liking. Percentage of starch affected only crunchy attributes. In addition, the interaction of different rice and starch (R\*S) did not affect any attributes. The interaction of different rice and percentage of starch (R\*P) affected color attribute. Different type of starch and different percentage of starch (S\*P) affected all attributes except overall flavor. However, the interaction of different varieties of rice, starch and percentage of starch (R\*S\*P) did not affect all attributes.

**Table 3:** P value on sensory characteristics of snack made from different variety of rice, starches and starch percentage [Rice (Jasmin rice, Brown rice and Riceberry); Starch (Corn starch and potato starch) and starch percentage (5% and 10%)]

	Color	Overall flavor	Crunchiness	Hardness	Chewiness	Overall Liking
Rice (R)	$< 0.001^*$	$0.4285^{ns}$	$0.1758^{ns}$	$0.4641^{ns}$	$0.8447^{ns}$	$0.5552^{ns}$
Starch (S)	$0.4147^{ns}$	$0.04430^*$	$0.2259^{ns}$	$0.1231^{ns}$	$0.1435^{ns}$	$0.0419^*$
Percentage (P)	$0.2158^{ns}$	$0.6753^{ns}$	$0.0046^*$	$0.1094^{ns}$	$0.1435^{ns}$	$0.3391^{ns}$
R*S	$1.0000^{ns}$	$0.9425^{ns}$	$0.9096^{ns}$	$0.6108^{ns}$	$0.6895^{ns}$	$0.8318^{ns}$
R*P	$0.0218^*$	$0.3557^{ns}$	$0.4302^{ns}$	$0.2595^{ns}$	$0.0814^{ns}$	$0.5418^{ns}$
S*P	$0.0024^*$	$0.1476^{ns}$	$0.0095^*$	$0.0245^*$	$0.0107^*$	$0.0029^*$
R*S*P	$0.1557^{ns}$	$0.8115^{ns}$	$0.7296^{ns}$	$0.5077^{ns}$	$0.8431^{ns}$	$0.8523^{ns}$

R=Rice, S = Starch, P = Percentage, ns = not significance, \* = significance

The human perception was used to evaluate sensory properties of food. However, the purpose of using machine was to determine the accuracy of the sensory test, if human perception was influenced by personal liking. Different type of starch affected the hardness of snack because different type of starch contains different ratio of amylose and amylopectin. Amylose affected the hardness whereas amylopectin affected the paste or gel formation.

Table 3 presented the hardness of rice-based snack measured by texture analyzer. There was significant difference in hardness ( $P < 0.05$ ). Snack from brown rice with corn starch (BR-5%CS, BR-10%CS) provided high score of hardness (due to bran layer around the kernel), and followed by Riceberry samples. This results was similar to the previous study reported by Sirichokworakit (2016). In contrast, the lowest score of hardness was observed in the samples made from Jasmine

rice (JR-5%PS). Jasmine rice showed unique characteristics of texture when it was cooked (soft). From the result showed in the table 3, it could be seen that the higher score of hardness was also observed in the samples made from Jasmine rice. The explanation is that the hardness may be resulted from the addition of corn and potato starch. When focusing on the samples that had highest score and lowest score of hardness, 10%PS gave highest score (1,313.27) and 5%PS gave lowest score of hardness (503.07) in the same sample made from Jasmine rice. Rice contains low content of amylose whereas corn contains higher content of amylose (Li *et al.*, 2007). The higher hardness was observed in the samples containing corn starch. The amylose in corn starch could affect the hardness of the pork-rice snack. The samples made from Brown rice and Jasmine rice with corn starch gained high score of hardness. On the other hand, the samples made from Riceberry gave high score of hardness with the formula contained 10%CS and 5%PS. The hardness was strongest in the sample made from JR-10%PS. In contrast, JS-5%PS obtained lowest score of hardness among other samples.

**Table 4:** Average score of hardness (g) (measured by texture analyzer) of snack made from different varieties of rice, starch, and starch percentage

Rice	Starch	%	Hardness	C.V.
Jasmine Rice	Corn Starch	5	1,017.51 <sup>abcd</sup>	0.24
		10	1,257.93 <sup>ab</sup>	0.20
	Potato Starch	5	503.07 <sup>c</sup>	0.14
		10	1,313.27 <sup>a</sup>	0.33
Brown Rice	Corn Starch	5	1,234.98 <sup>ab</sup>	0.29
		10	1,101.57 <sup>abc</sup>	0.03
	Potato Starch	5	678.37 <sup>de</sup>	0.17
		10	866.96 <sup>cd</sup>	0.18
Riceberry	Corn Starch	5	860.07 <sup>cd</sup>	0.29
		10	1,120.76 <sup>abc</sup>	0.15
	Potato Starch	5	1,249.50 <sup>ab</sup>	0.19
		10	893.94 <sup>bcd</sup>	0.47

Means ( $\pm$  Standard deviation) within a column followed by different letters are significantly different ( $P < 0.05$ )

According to P value (Table 5), hardness attribute might be affected by different type of starches, interaction of rice variety and type of starch (R\*S), interaction of rice variety and percentage of starch (R\*P) and interaction of rice variety, type of starch and percentage of starch (R\*S\*P).



**Table 5:** P value of hardness (measured by texture analyzer) of snack made from different varieties of rice, starch, and starch percentage.

	Hardness
Rice (R)	0.7166 <sup>ns</sup>
Starch (S)	0.0087 <sup>*</sup>
Percentage (P)	0.1297 <sup>ns</sup>
R*S	0.0182 <sup>*</sup>
R*P	0.0003 <sup>*</sup>
S*P	0.8266 <sup>ns</sup>
R*S*P	0.0028 <sup>*</sup>

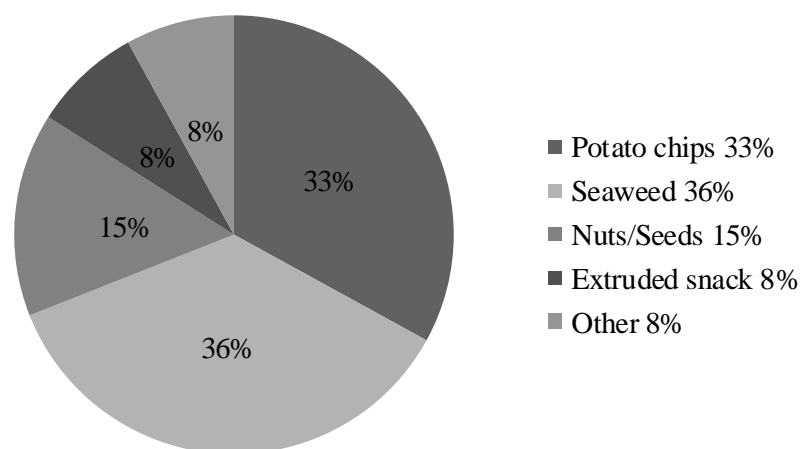
*R=Rice Types, S = Starch types, P = Percentage, ns = not significance, \* = significance*

### 3.3 Consumer survey

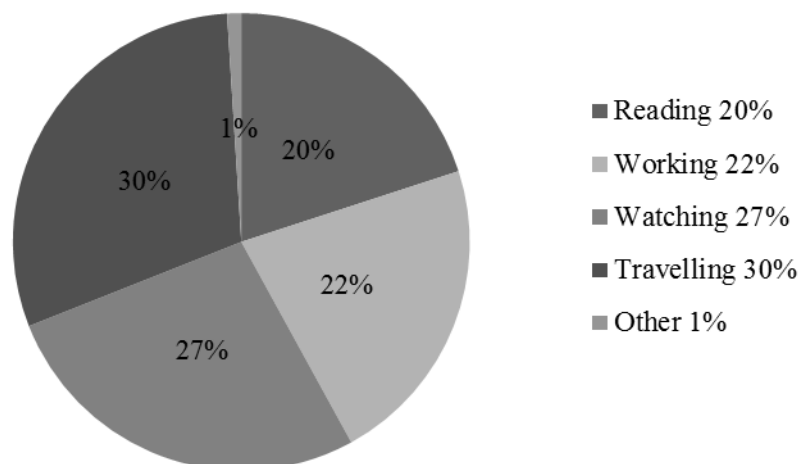
There were 2 potential formulas that can be developed to a final product: Jasmine rice with 5% corn starch and Brown rice with 5% corn starch as they obtained high preference score for overall attributes. However, only one formula (Brown rice with 5% corn starch) was developed as a final product for consumer survey in nutrient, texture and economic concern. Consumer's behavior, attitude, their needs towards the developed product, and general information of participated consumers were presented in Figure 1. 97% of consumers were familiar with the meat-based snack or snack containing meat. Savory taste or umami was one of the five basic tastes. Umami is the term that describes the taste of amino acid L-glutamate and nucleotides (5'-ribonucleotides such as guanosine monophosphate (GMP) and inosine monophosphate (IMP)). Disodium guanylate is a natural sodium salt enhancing nucleotide GMP produced from seaweed. Consumer liked seaweed snack and potato chips much more than nuts/seeds snack, extruded snack and other types of snack. The reason was that the GMP in seaweed snack enhanced the savory flavor. Similar to eastern cuisine, seaweed had been used as an ingredient for soup and other dishes in Japan to enhance the umami taste in the food. Therefore, consumer liked snack made from seaweed much more than the snack made from carbohydrate-based or sugar-based. Results also showed that 60% of the consumer ate snack every day, while 7% of the participants ate snack at least once a month. Only 2% of them did not consume snack. Snack had been consumed at any time including reading, watching, working and travelling. The majority of consumers bought snack from convenience store. Factors influencing consumer choices were dominated by packaging and taste and the result was shown in Figure 1d. The packaging, taste and quality were the most important factors that influence the choices of general snack. The distinctions of packaging and taste were at a significant level when it was compared with the other factors. Several consumers rated the promotion and availability as less important. Consumer rated price

of snack as moderately importance in this study, perhaps a reflection of the income level of the consumer.

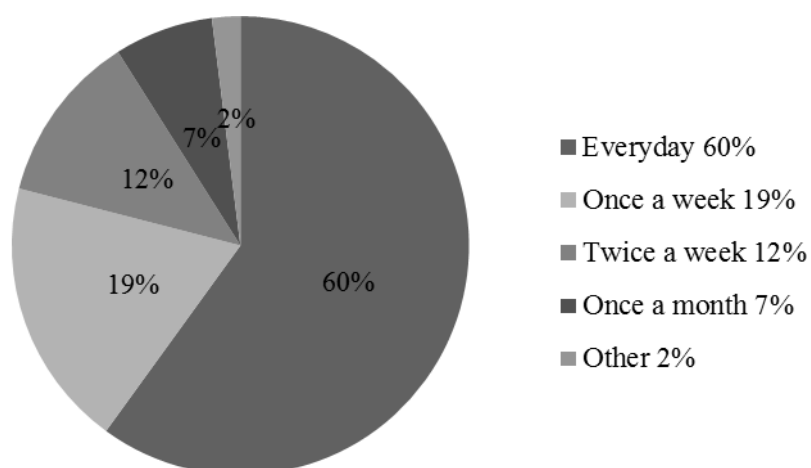
Panelists were asked to rate each attribute after testing the developed products and the result is shown in Table 5. The samples were rated ranged from 5.1 – 6.6 (neither like nor dislike to like slightly). The hardness obtained lowest score, while the color got highest score. Mean preference score of overall acceptance rated by participants to final product of pork-rice snack was 6.48 which means they were slightly like this product. 72% of the panelists accepted the product. 71% of panelist said they will buy pork-rice snack when it launches in the market. 55% of panelists desired to buy about 11-15 baht (50-g/pack). Over one-fourth of them did not want to buy this product. These results are consistent with findings in another research. Verbeke *et. al.*, (2011) indicated that female consumed pork less than male as they concern more about the physical body and the information of product when they decided to buy food. They thought that this snack should have been high in fat.



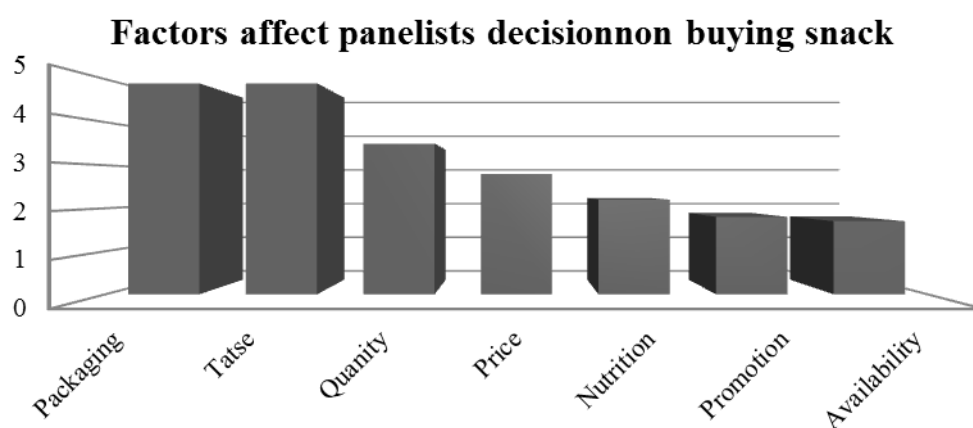
a) What types of snack do you like the most?



b) When do you consume snack?



c) How often do you consume snack?



d) What are the following factors affect your decision on buying snack?

**Figure 1:** Consumer behavior of snack in Thailand a) What types of snack do you like the most? b) When do you consume snack? c) How often do you consume snack? and d) What are the following factors affect your decision on buying snack?

**Table 5:** Liking score on 9-point hedonic preference test of pork-rice snack

Attributes	Preference scores
Appearance	6.39 ± 0.56
Color	6.64 ± 0.93
Sweetness	6.34 ± 0.51
Hardness	5.12 ± 1.87
Crispiness	5.30 ± 0.94
Overall acceptance	6.48 ± 2.28

## **4 CONCLUSIONS**

Sample with ratio of 30:70 of pork: rice was suitable for pork rice snack. Riceberry provided the best texture to the rice-based snack but the score of color was low due to anthocyanins in Riceberry that was dissolved easily in water and sensitive to heat, which affected to the liking toward snack of the panel. Brown rice also provided a good texture (hardness and crispiness) due to the bran layers on the Brown rice that helped to improve the texture of the rice-based snack. Using 5% corn starch was optimum in texture improvement of pork-rice snack.

There were 2 potential formulas that can be developed to a final product: Jasmine rice with 5% corn starch and Brown rice with 5% corn starch. Brown rice with 5% corn starch was chosen because of the nutrient, texture and economic reason. The consumer subjects were mostly female (88%), students (58%) and aged of 16-25. This age group is representative of snack consumer and approximately 70% of them consumed 1 time or more per week. The snack they like to consume was seaweed and potato chips. Consumers consuming the nuts/seeds and extruded snack were fewer. From the consumer survey, it was found that consumer slightly like the final product and they would buy the snack at the price of 11-15 baht.

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## **DEVELOPMENT OF VEGETABLE-RICH KAENG LIANG CUBE**

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### **ABSTRACT**

To increase the nutritive value, dried pumpkin was added into the Hairy basil-rich Kaeng Liang cube. Pumpkin was cut into small pieces and dried at 40°C for 5 hours. The ingredients of Hairy basil-rich Kaeng Liang cube were shallot, fingerroot, black pepper, fried shrimp, shrimp paste, and dried hairy basil. Amount of dried pumpkin addition was varied by sharing with the amount of dried hairy basil addition which is 13.0% from the basic formula. The vegetable-rich Kaeng Liang cube was developed by varying the ratio between dried pumpkin and dried hairy basil at 50:50, 60:40, 70:30 and 80:20 and sensory evaluation was done by 40 panellists. As the results, the final formula of vegetable-rich Kaeng Liang cube consist of shallot 13.3%, fingerroot 6.8%, black pepper 10.8%, dried shrimp 33.5%, shrimp paste 22.5%, chili 1.3%, dried hairy basil 4.7% and dried pumpkin 7.1%. For the proximate analysis, the amount of fat, ash, moisture, protein, and carbohydrate were 2.6, 14.3, 21.3, 30.5, and 31.3%, respectively, while fiber content was 18.0%. The microbial analysis by 3M Petrifilm™ showed that the product was safe from total coliform bacteria, *E.coli*, and yeast and mold.

**Keywords:** Kaeng Liang, Thai curry, Thai food, Herbs, Spices

### **1 INTRODUCTION**

Recently, traditional Thai foods are of interest due to consumers' health concerns. Many local Thai foods contained high amount of vegetables, fruits, herbs, and spices either fresh or cooked form [1,2]. Thai local vegetables comprised more than 150 species of plants, consuming either fresh or cooked vegetables and some are used as ingredients in Thai cuisine [3]. For Thai foods, ingredients composition of identical name of dish may differ from home to home or region to region [4]. Moreover, the difference of spices used in cooking style usually depend on protein base used as red meat, goat, sheep, beef or buffalo, or white or light meat as chicken and seafood [5].

Herbs and spices are always found in Thai cooking [6, 7]. Kaeng Liang or spicy mixed vegetable soup is one of the oldest cuisines in Thailand which contained low fat, low calories, and rich of

fibers from various vegetables which may differ from home to home or region to region [4]. To make Kaeng Liang soup, Kaeng Liang paste should be prepared. Kaeng Liang paste is an aromatic mixture of pepper, shallot, fingerroot, chili, kapi or shrimp paste and dried shrimp or dried fish [1, 2, 8, 9]. This paste has a key role in Kaeng Liang soup because it is the main component.

Normally, Kaeng Liang soup is added many local vegetables such as pumpkin, gourds, angle luffa, baby corn, straw mushroom, ivy gourd leaves, and Hairy basil or Lemon basil [1, 2] depending on the area and the individual. The unique taste came from pepper, chili, fingerroot and shallot in the paste, while the unique flavor and aroma came from the Hairy basil and fingerroot. Those herbs and spices including vegetables provided not only the unique flavor for the soup but also more benefit to health. Not only the spices and herbs but also kapi or shrimp paste enriched the antioxidants. Kapi is a Thai traditional fermented shrimp paste producing by fermenting small shrimp (*Acetes vulgaris*) or krill (*Mesopodopsis oreintalis*). Recently, it has been reported that fermented shrimp and krill products exhibited strong antioxidant activities [10]. Therefore, Kaeng Liang is considered as one of functional foods which contain high antioxidants.

Spices and herbs are reported to contain bioactive compounds imparting antioxidant [5, 7, 11], preservative [7, 12], antimicrobial properties [13, 14], and have medicinal effects to the food [15]. Being natural foodstuffs, spices and herbs appeal to many consumers who question the safety of synthetic food additives. Some spices and herbs used today are valued for their antimicrobial activities and medicinal effects in addition to their flavor and fragrance qualities, including processed or raw food preservation, pharmaceuticals, alternative medicines and natural therapies [15]. It has been reported that the ingredients of Kaeng Liang paste are the good sources of phenolic compounds, such as chili, pepper [7, 16, 17], and shallots [18], while it found flavonoid rich in fingerroot [19, 20]. A highly positive linear relationship exists between antioxidant activity and total phenolic content in some spices and herbs [15, 17].

Pumpkin and Hairy basil are two important herbs commonly adding into Kaeng Liang soup. Pumpkins are gourd squashes of the genus *Cucurbita* and the family *Cucurbitaceae* which are cultivated worldwide [21]. Pumpkin is an important medicinal plant, which contains a large amount of water (94% of its weight) [22]. Pumpkin flesh also contains moderate content of carbohydrates, vitamins, and minerals [23] including other substances beneficial to human health such as antioxidants, tocopherols, carotenoids,  $\gamma$ -aminobutyric acid but low in fat and calories [21, 24]. Moreover, this plant is considered as a good treatment for diabetes and can prevent changes in plasma lipids and blood pressure associated with inadequate estrogen availability [22].

Hairy basil (*Ocimum basilicum*) is the plant of genus *Ocimum* L. (Lamiaceae) which has a spicy, lemony taste with fruity aroma [25]. It is well known for its use in cooking, however, the herb is also noted for its value in traditional therapeutic potentials [26, 27]. Hairy basil has been reported to



have digestive, antifungal, antibacterial, anti-melanoma, radioprotective, antimicrobial, anthelmintic, and antioxidant capacity as phenolic acids especially tannin and flavonoids [25, 27, 28, 29].

In modern lifestyle, there are smaller families with less in-home cooking activity. Ready-to-cook convenient foods such as marinated products are becoming quite popular among these groups of consumers [6]. Commercially prepared instant soups such as canned, dehydrated, and frozen soups have replaced homemade soups as preparing the soup with good appeal is a time-consuming process. Dry soup mixes or soup pastes containing vegetables (or meat) in many forms are second in popularity only to canned soup. Any soup mix or paste should be easy to cook within a minimum time and should be nutritious and palatable as canned or frozen products [30].

Many Thai curry or Thai style spicy soups consumed the preparing time. Recently, there are many commercial curry pastes or instant curry powders as a condiment available in the market such as Thai green curry paste [6, 15], Thai red curry powder [7, 15], instant Tom Yum curry, or Massaman curry paste [31]. However, it takes a long preparing time to prepare other ingredients especially vegetables and meat to make curry or soup. Thus, for Thai people who stay in the big city, the homemade meals are rarely seen and are being replaced by ready-to-cook and ready-to-eat foods bough at local markets, supermarket standalone, food stalls, grocery store, convenience store, supermarkets or big department stores and eaten at home [31].

Kaeng Liang is another Thai food that needs many steps to prepare especially during the step of Kaeng Liang paste preparation. Therefore, the objectives of this research were to develop the vegetable-rich Kaeng Liang cube having enriched nutrients, taste, flavor, and aroma of ingredient used.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Pepper powder (*Piper nigrum* Linn.), hairy Basil (*Ocimum canum* Sims.), shallot (*Allium ascalonicum* Linn.), fingerroot (*Boesenbergia pandurata* Holtt.), chili (*Capsicum minimum* Roxb.), Pumpkin (*Cucurbita maxima*), shrimp paste, and dried shrimp were bought from local market, while 3M Petrifilm™ E.coli/Coliforms Count Plate and 3M Petrifilm™ Yeast and Mold Count Plate were supported rom 3M Thailand Co., Ltd.

### **2.2 Formulate the Hairy basil-rich Kaeng Liang cube**

Sliced shallot, sliced fingerroot, and hairy basil leaves were dried using tray dryer at 40°C for 5 hours, and then roughly ground. As basic formula (dry basis) shown in table 1, dried hairy basil leaves powder was added into Kaeng Liang cube at 5, 10 and 15% (dry weight). All ingredients were mixed and formed into cube shape by using ice cube maker. Kaeng Liang cubes were dried at

40°C for 2 hours. Kaeng Liang soup was prepared by boiled with 150 mL of water before evaluated aroma, color, taste, and overall liking by 20 trained panelists using 9-point hedonic scales.

**Table. 1:** Basic formula of Kaeng Liang cube (dry basis)

Ingredients	Percentage (%)
Dried shallot powder	9.9
Dried fingerroot powder	5.1
Black pepper powder	8.1
Dried shrimp powder	25.0
Shrimp paste	10.0
Chopped chili	1.0

### **2.3 Investigate the effect of shrimp paste content on the sensory of hairy basil-rich Kaeng Liang cube**

Hairy basil-rich Kaeng Liang cube was prepared as the formula from the previous step. Dried ground shallot, dried ground fingerroot, black pepper powder, blended dried shrimp, and chopped chili were mixed with shrimp paste at 5, 10, and 15 % (w/w). The mixed ingredients were formed into cube shape and dry at 40°C for 2 hrs. Kaeng Liang soup was prepared by boiled with 150 mL of water before evaluated by 20 trained panelists using 9-point hedonic scores.

### **2.4 Study the effect of dried pumpkin adding on the sensory of hairy basil-rich Kaeng Liang cube**

Dried pumpkins were added at various contents into the hairy basil-rich Kaeng Liang cube by sharing with the amount of dried hairy basil addition. The ratio of dried pumpkin and dried hairy basil was 50:50, 60:40, 70:30 and 80:20. Vegetable-rich Kaeng Liang cube was boiled with 150 mL of water to make soup and evaluated the preference of 30 panelists using 9-point hedonic scale.

### **2.5 Investigate the chemical and microbiological properties of vegetable-rich Kaeng Liang cube**

#### *(1) Proximate analysis*

Vegetable-rich Kaeng Liang cube was analyzed the chemical properties by proximate analysis that are ash content, fat content, moisture content, protein content, and carbohydrate content including fiber content [32].

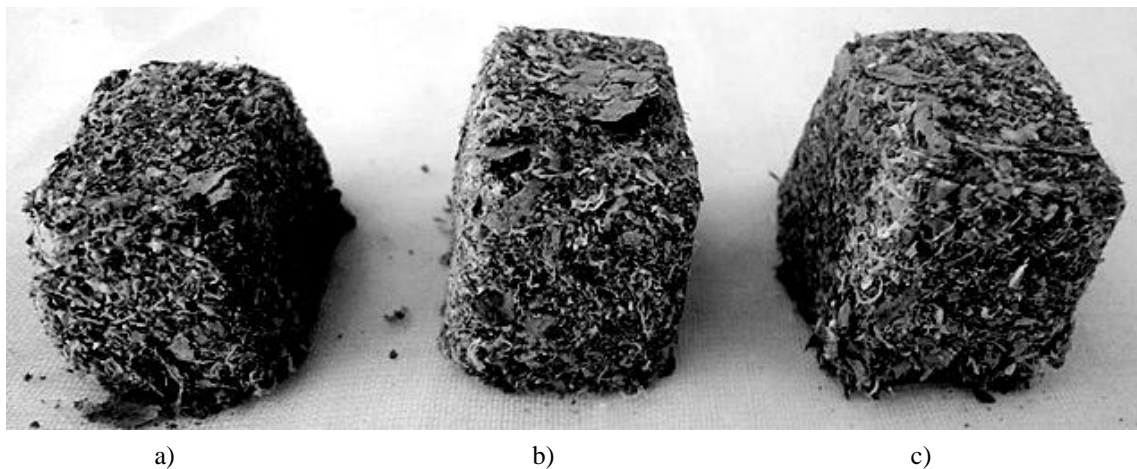
#### *(2) Microbial analysis*

Vegetable-rich Kaeng Liang cube 25 g was mixed with 250 mL of 0.1% peptone and homogenized for 2 minutes. Sample was investigated the Coliform, *E. coli*, and Yeast and mold by using 3M™ Petrifilm™.

### 3 RESULTS AND DISCUSSION

#### 3.1 Formulate the Hairy basil-rich Kaeng Liang cube

Kaeng Liang cube was developed using basic formula from previous study. The ingredients consisted of dried shallot powder 9.9%, dried fingerroot powder 5.1%, black pepper powder 8.1%, dried shrimp flake 25%, shrimp paste 10%, and chopped chili 1%. To increase the nutrition value and enhance its unique flavor and aroma, dried hairy basil was added at 5, 10 and 15% (dry weight) as one of ingredients. For the dried hairy basil at higher than 15% (w/w), all ingredients could not form cubic shape according to the unsuitable ratio between dried ingredients and shrimp paste. Kaeng Liang cube was dried at 40°C for 2 hours before kept in plastic airtight container. The weight of dried paste cube was approximately 10 grams. Dried paste cube was boiled with 150 mL of water to make soup. Kaeng Liang soup was evaluated aroma, color, taste, and overall liking by 20 trained panelists using 9-point hedonic scale.



**Figure 1:** Different Kaeng Liang cube adding various amounts of dried hairy basil; (a) 5%, (b) 10% and (c) 15% (w/w)

**Table. 2:** Sensory evaluation score of Kaeng Liang cube with various amount of dried hairy basil

% Hairy basil (w/w)	Attribute			
	Aroma	Color	Taste	Overall liking
5	5.7 ± 0.9 <sup>a</sup>	5.9 ± 0.7 <sup>a</sup>	6.2 ± 0.8 <sup>a</sup>	6.1 ± 0.7 <sup>a</sup>
10	7.1 ± 0.5 <sup>b</sup>	6.7 ± 1.1 <sup>b</sup>	6.6 ± 0.9 <sup>b</sup>	6.9 ± 0.5 <sup>b</sup>
15	7.8 ± 0.6 <sup>c</sup>	7.4 ± 0.9 <sup>c</sup>	7.7 ± 0.7 <sup>c</sup>	7.8 ± 0.6 <sup>c</sup>

As the results shown in table 2, dried hairy basil at 15% (w/w) showed higher score for all attributes than 10 and 5% (w/w), respectively ( $P \leq 0.05$ ). Those results implied that dried hairy basil at 15% (w/w) was the highest amount suitable to add into Kaeng Liang cube. The score of

15% dried hairy basil in Kaeng Liang cube for aroma, color, taste, and overall liking were  $7.8 \pm 0.6$ ,  $7.4 \pm 0.9$ ,  $7.7 \pm 0.7$  and  $7.8 \pm 0.6$ , respectively. Therefore, the ingredients for the final formula of hairy basil-rich Kaeng Liang cube consisted of shallot 14.6%, fingerroot 7.5%, black pepper 11.8%, dried shrimp 36.9%, shrimp paste 14.7%, chili 1.5%, and dried hairy basil 13% (w/w).

### 3.2 Investigate the effect of shrimp paste content on the sensory of hairy basil-rich Kaeng Liang cube

From the previous study, even though, the increasing of hairy basil content in Kaeng Liang cube did not affected to taste and flavor of Kaeng Liang soup ( $P \geq 0.05$ ), there was some comments from panelists that this changing of taste and flavor resulting from shrimp paste. The effect of shrimp pastes on the taste and flavor of Kaeng Liang soup came from the progress of fermentation during storage. Shrimp paste was bought in a small bottle. It was opened and used in small amount, and then kept as stock. Before shrimp paste in stock bottle finished off the rest, the quality of shrimp paste changes. Faithong and Benjakul (2014) reported that the fermentation of shrimp paste was generally taken place until the typical aroma was developed and during fermentation, enzymatic hydrolysis yielded a large amount of short chain peptide and amino acid [10]. With increased fermentation, the degree of hydrolysis and amino nitrogen content increased resulting the increasing of more flavor intensity and salty taste.

Thus, the amount of shrimp paste was investigated its effect on the Kaeng Liang soup. The hairy basil-rich Kaeng Liang cube was added less shrimp paste from the original amount (14.7%) at 5, 10, and 15 % (w/w) to reduce the salty taste and strong shrimp paste flavor in Kaeng Liang soup.

**Table 3:** Sensory evaluation scores of hairy basil-rich Kaeng Liang cube with various shrimp paste content

Attributes	% Shrimp paste (w/w)		
	5%	10%	15%
Color	$6.2 \pm 0.9^{ab}$	$6.6 \pm 1.4^b$	$6.1 \pm 1.2^a$
Aroma	$6.1 \pm 1.1^a$	$7.3 \pm 1.4^b$	$6.1 \pm 1.2^a$
Flavor	$6.1 \pm 1.1^a$	$7.3 \pm 1.3^b$	$6.1 \pm 1.3^a$
Taste	$6.2 \pm 1.2^a$	$7.6 \pm 1.2^b$	$6.4 \pm 1.3^a$
Overall liking	$6.1 \pm 1.2^a$	$7.1 \pm 1.4^b$	$6.3 \pm 1.3^a$

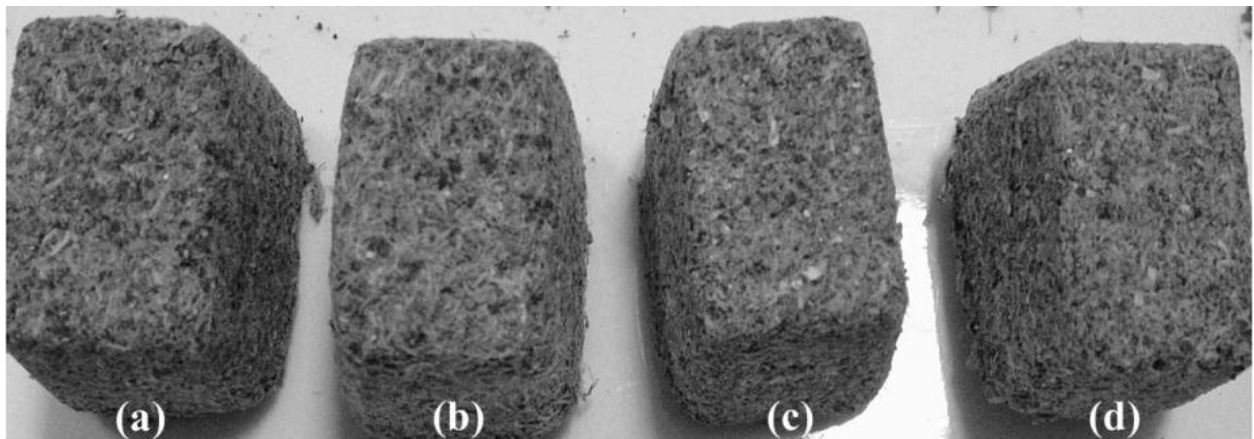
From table 3, the results distinctly show that the Kaeng Liang cube with 10% shrimp paste has the highest mean score for all attributes which are color, aroma, flavor, taste and overall liking. The scores of this formula are  $6.6 \pm 1.4$ ,  $7.3 \pm 1.4$ ,  $7.3 \pm 1.3$ ,  $7.6 \pm 1.2$  and  $7.1 \pm 1.4$  for color,

aroma, flavor, taste and overall liking, respectively. There was no significant difference ( $p \geq 0.05$ ) between color of Kaeng Liang cube with 5 and 10% shrimp paste and between 10 and 15% shrimp paste. For other attributes (aroma, flavor, taste and overall liking), Kaeng Liang cube with 10% shrimp paste was significantly different ( $p \leq 0.05$ ) from other formulas. Hence, hairy basil-rich Kaeng Liang cube with shrimp paste 10% (w/w) was selected for further development.

Then, the selected formula was converted its ingredients in 100% ratio as standard formula. The converted formula consisted of dried shallot powder 13.3%, dried fingerroot powder 6.8%, black pepper powder 10.8%, dried shrimp powder 33.5%, shrimp paste 22.5%, chopped chili 1.3%, and dried hairy basil powder 11.8% (w/w).

### **3.3 Study the effect of dried pumpkin on the sensory of hairy basil-rich Kaeng Liang cube**

Dried pumpkins were added at various contents into the hairy basil-rich Kaeng Liang cube by sharing with the amount of dried hairy basil powder added into Kaeng Liang cube which is 11.8% from the standard formula.



**Figure 2:** vegetable-rich Kaeng Liang cube with various ratio of dried pumpkin and dried hairy basil powder; (a) 50:50, (b) 60:40, (c) 70:30 and (d) 80:20

To determine the suitable ratio of dried pumpkin and dried hairy basil powder, the penalty analysis method was applied. Penalty analysis is a method used in sensory analysis to identify potential directions for the improvement of products based on surveys performed on consumers. It has been used extensively by practitioners in the industry to assist in identifying decreases in acceptability associated with sensory attributes not at optimal levels in a product [33]. Therefore, the ratio of dried pumpkin and dried hairy basil powder were varied at 50:50, 60:40, 70:30, and 80:20 (table 4). Vegetable-rich Kaeng Liang cube was prepared and evaluated the preference of its soup.

**Table 4:** Vegetable-rich Kaeng Liang cube with the variation ratio of dried hairy basil powder and dried pumpkin

Ratio	Dried pumpkin (% w/w)	Dried hairy basil powder (% w/w)
50:50	5.9	5.9
60:40	7.1	4.7
70:30	8.3	3.5
80:20	9.4	2.4

**Table 5:** Sensory evaluation of vegetable-rich Kaeng Liang cube with various ratios of dried hairy basil powder and dried pumpkin

Ratio of dried pumpkin : dried hairy basil powder	Aroma	Taste	Overall liking
50:50	7.1 ± 0.9 <sup>b</sup>	6.5 ± 0.7 <sup>ab</sup>	7.0 ± 0.6 <sup>b</sup>
60:40	7.4 ± 0.8 <sup>b</sup>	7.8 ± 0.8 <sup>c</sup>	7.5 ± 0.5 <sup>c</sup>
70:30	7.3 ± 0.8 <sup>b</sup>	6.6 ± 0.8 <sup>b</sup>	7.1 ± 0.7 <sup>b</sup>
80:20	6.6 ± 0.7 <sup>a</sup>	6.1 ± 0.9 <sup>a</sup>	6.4 ± 0.7 <sup>a</sup>

As the results shown in table 5, the highest score for aroma, taste, and overall liking showed at the vegetable rich-Kaeng Liang cube with the ratio of dried pumpkin and dried hairy basil powder at 60:40 or 3:2 ( $P \leq 0.05$ ). For the aroma, there was no significantly different ( $p \geq 0.05$ ) between the ratios of 50:50, 60:40, and 70:30 but the ratio of 80:20 was significantly different ( $p \leq 0.05$ ) from others. For taste and overall liking, the ratios of 50:50 and 70:30 was not significantly different from each other but the ratio of 60:40 and 80:20 was significantly different from all others. The ratio of 80:20 showed the lowest score for all attributes due to the high amount of dried pumpkin and its aroma stronger than aroma of hairy basil. Thus, the ratio 60:40 with the highest score of all attributes was selected as product prototype for further analysis.

In Table 6, the final formula of vegetable-rich Kaeng Liang cube consisted of dried shallot powder 13.3%, dried fingerroot powder 6.8%, black pepper powder 10.8%, dried shrimp powder 33.5%, chopped chili 1.3%, shrimp paste 22.5%, dried hairy basil powder 4.7% and dried pumpkin 7.1% (w/w).



**Table 6:** Final formula of vegetable-rich Kaeng Liang cube

<b>Ingredient</b>	<b>% (w/w)</b>
Dried shallot powder	13.3
Dried fingerroot powder	6.8
Black pepper powder	10.8
Dried shrimp powder	33.5
Shrimp paste	22.5
Chopped chili	1.3
Dried hairy basil powder	4.7
Dried pumpkin	7.1

### 3.4 Investigate the chemical and microbiological properties of vegetable-rich Kaeng Liang cube

Vegetable-rich Kaeng Liang cube was analyzed the chemical properties by proximate analysis to investigate the nutrition value. Proximate analysis is the investigation of ash content, fat content, protein content, and moisture content, however, the carbohydrate content get from calculation. As the results, vegetable rich-Kaeng Liang cube contained ash 14.3%, fat 2.6%, moisture content 21.3%, protein 30.5% and carbohydrate 31.2% (table 7). Moreover, the prototype product also measured amount of dietary fiber according to the vegetable rich in ingredients. The amount of dietary fiber of vegetable-rich Kaeng Liang cube was 18% implying that the vegetable-rich Kaeng Liang cube could be claimed as high dietary fiber product.

**Table 7:** Proximate analysis of vegetable-rich Kaeng Kiang cube

<b>Analysis</b>	<b>%</b>
Ash	14.3
Crude fat	2.6
Moisture content	21.3
Protein	30.5
Carbohydrate	31.3

To study the microbial property, vegetable-rich Kaeng Liang cube 25 g was prepared by mixing with 250 mL of 0.1% peptone solution. The sample solution was diluted to  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$  to reduce the density of microorganisms before investigated the contamination of *E. coli*,

Coliforms, and Yeast and Mold using 3M Petrifilm™. The 3M Petrifilm™ *E.coli*/Coliforms Count Plate was used to identify both *E. coli* and other Coliforms with confirmed results after incubated at 35±1°C in 24-48 hours. For yeast and mold count, 3M Petrifilm™ Yeast and Mold Count Plate was used to differentiate and enumerate yeast and mold. After inoculated, the plates were incubated at 20-25°C for 3-5 days.

As the results, vegetable-rich Kaeng Liang cube had *E.coli*, total coliform, yeast and mold less than 30 cfu/g of food, while the Notification of the Ministry of Public Health (no.210) B.E.2000 said that *E.coli* and mold should be found less than 3 and 100 in 1 g of food. This implied that the vegetable-rich Kaeng Liang cube was safe from *E.coli*, total coliform, yeast and mold because raw materials used were dried materials except shrimp paste. However, shrimp paste contained high salt content (approximately 16 to 20% w/w) and had low water activity [10]. Moreover, finished vegetable-rich Kaeng Liang cube was dried for 2 hours before kept in the air-tight packaging. Thus, this step could be guaranteed for dried characteristic of the product and there was very rare chance to spoilage with microorganism especially *E.coli* and total coliforms, and yeast and mold.

#### 4 CONCLUSIONS

Vegetable-rich Kaeng Liang cube consisted of shallot 13.3%, fingerroot 6.8%, black pepper 10.8%, dried shrimp 33.5%, shrimp paste 22.5% , chili 1.3%, dried hairy basil 4.7% and dried pumpkin 7.1%. The proximate analysis that is crude fat, ash, moisture, protein, and carbohydrate were 2.6, 14.3, 21.3, 30.5, and 31.3%, respectively, while fiber content was 18.0%. The microbial analysis by 3M Petrifilm™ showed that the product was safe from Coliforms, *E.coli*, and yeast and mold.

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## DETERMINATION OF BIOCHEMICAL COMPOSITION AND IN VITRO DIGESTIBILITY OF PROTEIN ISOLATE FROM BRACKISH ALGAE *CHAETOMORPHA* SP.

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### ABSTRACT

Difference on protein quality is related to processing condition and biochemical composition of algae protein isolate (API). Removing lipid and carbohydrate increased protein content and protein digestibility of API. API from *Chaetomorpha* sp. contained 72.5% w/w protein and exhibited high digestibility (83.8%), similar ( $p>0.05$ ) to soy protein isolate and lower ( $p<0.05$ ) than that of caseinate isolate supplements, which were used as reference. According to the WHO/FAO/UNU protein standard for non-athletic adult, the API from *Chaetomorpha* sp. scored high AAS, the PDCAAS values were  $>1.0$ . However, when the calculated AAS and PDCAAS based on the suggestion for children were considered, API from *Chaetomorpha* sp. exhibited suboptimal score values for several EAA and was unable to supply the suggested children EAA requirement.

**Keywords:** *Chaetomorpha* sp., algae protein isolate (API), biochemical composition, in vitro digestibility

### 1 INTRODUCTION

Growing fast and habiting in seawater and brackish water, green algae are easily collected. They also can be co-cultured with shrimp in brackish water shrimp ponds in Mekong Delta. The algae biomass from *Chaetomorpha* sp. is rich in protein (12-21% w/w db) [9]. Containing high amount of protein with balanced profile of amino acid, protein isolate derived from *Chaetomorpha* sp. biomass can play significant roles in human nutrition, particularly in developing countries where average protein intake is less than that required.

Differences in the biochemical composition of algae protein isolate potentially influence its nutritional effect on the human body [4, 13]. The nutritional quality of algae protein depends on amino acid composition, bioavailability of essential amino acids, protein digestibility, and

physiological utilization of specific amino acids after digestion and absorption [2, 6]. Moreover, protein digestibility is an important factor to estimate the protein availability for intestinal absorption after digestion reflecting on the efficiency of protein utilization on diet [18].

Several reports have investigated the extraction of protein from marine algae [3, 7, 10, 13] and properties of seaweed protein [1, 8, 19]. However, there is very limited information regarding the utilization of protein from brackish green algae, especially quality of protein from *Chaetomorpha* sp. Therefore, the objective of this study was to determine the composition of algae protein isolates (API) from *Chaetomorpha* sp. and to investigate their protein quality, evaluating the in vitro protein digestibility, and the essential amino acid (EEA) composition. In addition, the amino acid score (AAS) and protein digestibility-corrected amino acid scores (PDCAAS) were also calculated.

## 2 MATERIAL AND METHODS

### 2.1 Material

Fresh brackish green algae (*Chaetomorpha aerea*, *Chaetomorpha linum*, and *Chaetomorpha gracilis*) were harvested from shrimp ponds in Bac Lieu province. Algae samples were washed with fresh water to remove snails and foreign objects like sand. Dried algae were obtained by drying fresh algae at 50-60°C to reach 7-8% w/w moisture content.

### 2.2 Preparation of Algae Protein Isolate (API)

Dried algae biomass was ground with 80 mesh screen to fine powder. Biomass was then extracted by using de-ionized water adjusted to pH 10-11 with 1 N NaOH (biomass: solvent ratio 1:20 w/v), at 50°C for 1h to obtain API1. To obtain API2, lipid, wax and chlorophyll were removed from biomass by diethyl ether extraction. Defatted biomass was incubated with cellulase Crestone Conc. of Genecor (substrate concentration 10% w/v, enzyme concentration 100UI/g db, at 50°C pH 7 for 2 hrs). 1N NaOH solution and de-ionized water were then added to reach biomass: solvent ratio 1:20 w/v, pH 10-11. The mixture was incubated at 50°C for 1h to extract protein.

The slurry was then centrifuged at 10,000 g for 30 min at 4°C to separate algae biomass residue. The protein containing supernatant was acidified by 1N HCl solution to pH 4.0-4.5 (algae protein isoelectric point) for protein precipitation. After centrifugation, protein precipitate was washed with water, re-dissolved in water, neutralized to pH 7 with 1N NaOH at room temperature, and then freeze-dried to yield protein powder.

### 2.3 Proximate Analysis

Moisture, fat and ash contents were determined according to the methods of AOAC 2012 numbers 950.46, 960.39 and 920.153 respectively. The protein content of sample was determined by the Kjeldhal method. Total carbohydrate was determined using analytical methods described

by Dien B.S. (2010) [5]. Dried materials were hydrolyzed using sulfuric acid in two stages. The hydrolysis conditions were acid concentration of 72% (v/v) at 30°C and 3.6% (v/v) at 120°C for the first and second stage, respectively. The hydrolysis duration time was 1 h for both stages. The hydrolysate was analyzed for monomeric sugars released by HPLC using Aminex HPX 87P column and RI detector. The contents were expressed on a dry weight basis. Each analysis was performed in triplicate, and data was reported as mean  $\pm$  standard deviation.

#### **2.4 In vitro protein digestibility (IVPD) assay**

The *in vitro* protein digestibility was evaluated based on method described by Water Akeson and Stahmann (1964) [17] with modifications [2]. Briefly, aliquots of 250 mg of each sample or 250 mL of deionized water (for the blank) were suspended in 15mL of 0.1 mol equi/L HCl containing 1.5 mg/mL pepsin (Sigma®, St. Louis, MO, USA), and incubated for 3 h at 37°C in a water bath. The pepsin hydrolysis ceased after neutralization with the addition of 7.5 mL of 0.5 mol equi/L of NaOH. Then, the pancreatic digestion was initiated with the addition of 10mL of 0.2 mol/L phosphate buffer (pH8.0) containing 10 mg of pancreatin (Sigma®, St. Louis, MO, USA) with 1 mL of 0.005 mol/L sodium azide to prevent microbial growth, and the mixture was incubated at 37°C overnight. After the pancreatic hydrolysis, 1 mL of 10 g/100 mL of trichloroacetic acid was added, followed centrifugation at 5000g for 20 min. The supernatant was collected, and the total protein content was estimated based on the nitrogen content using Kjeldahl AOAC method 930.29 (AOAC, 2012). For comparative purpose, supplements manufactured with soy protein and caseinate isolate powder were used as references. The IVPD values were calculated according to the equation:

$$\% \text{ Digestibility} = (N_s - N_b)/N_s \times 100$$

Where  $N_s$  and  $N_b$  represent the nitrogen content in the supernatant and in the sample, respectively.

#### **2.5 Determination of essential amino acids (EAA) content**

The essential amino acids (histidine, threonine, methionine, valine, phenylalanine, isoleucine, leucine and lysine) content were analyzed by high-performance liquid chromatography, using method described by Almeida C.C (2015) [2]. Briefly, 50 mL of sample previously diluted according to the manufacturer's recommendation, was mixed with 50 mL of 1.5 mol/L perchloric acid (v/v). After 2 min at room temperature, 1.125 mL of ultrapure water and 25 mL of 2 mol/L potassium carbonate were added. The tubes were centrifuged at 10,000 g for 1 min, and then, 100 mL of the supernatant was diluted with 100 mL of 1.2g/100mL benzoic acid and 1.4 mL of ultrapure water. The amino acids were identified by using a pre-column derivation with o-phthalaldehyde (Sigma®, St. Louis, MO, USA). The HPLC instrument was equipped with a quaternary pump (Agilent 1260 Infinity LC, USA), a 5 mm reverse-phase C18 column (4.6 mm ID 150 mm from Supelco®, Bellefonte, PA, USA) guarded by a 5 mm reverse-phase C18 guard column Ascentis® (4.0 mm ID 20 mm from Sigma®, Bellefonte, PA, USA), and a fluorescence

detector (Agilent 1260 Infinity LC, USA) monitoring excitation and emission wavelengths at 340 nm and 455 nm, respectively. The samples were separated by mobile phase gradient using 0.1 mol/L sodiumacetate (pH 7.2), and methanol at 1.1 mL/min flow. The total running time per sample was 49 min and the column temperature was kept at room temperature.

## 2.6 Amino acid score (AAS) and protein digestibility-corrected amino acid score (PDCAAS)

The AAS was calculated by dividing each individual amino acid content by their respective reference value, considering the daily amino acid requirement for non-athletic adults (Joint WHO/ FAO/UNU Expert Consultation, 2007) and 1-2 year old children (Joint WHO/ FAO/UNU Expert Consultation, 2007 [18]).

The PDCAAS were calculated by multiplying the AAS value of each essential amino acid by the protein digestibility.

## 2.7 Statistical Analysis

The data was analyzed by Analysis of Variance (ANOVA). Least Significant Differences (LSD) were calculated at  $p < 0.05$  to compare treatment means using the Statgraphics Centurion XV (StatPoint Inc., USA) software for Windows 8.

# 3 RESULT AND DISCUSSION

## 3.1 Chemical Characterization of Algae Protein Isolate (API)

The proximate composition of two products API1 and API2 are shown in Table 3.1.

**Table 3.1:** Chemical compositions of Algae Protein Isolate API1 and API2

Samples	API1	API2
Moisture	$7.1 \pm 0.45^{b1}$	$8.5 \pm 0.68^{a1}$
Ash	$6.1 \pm 0.21^{a2}$	$5.9 \pm 0.37^{a2}$
Lipid + ether extractables	$8.9 \pm 0.52^{a3}$	$0.78 \pm 0.01^{b3}$
Protein	$53.2 \pm 1.78^{b4}$	$72.5 \pm 2.45^{a4}$
Carbohydrate	$15.5 \pm 1.15^{a5}$	$2.15 \pm 0.21^{b5}$

*Results represent the average of three determinations  $\pm$  SD, values in the same raw with different letters are significantly different ( $p < 0.05$ )*

API1 contained 8.9% fat and ether extractables (wax, chlorophyll...) while API2 contained only 0.78%. It was indicated that defatted procedure used in API2 production could reduce the fat and



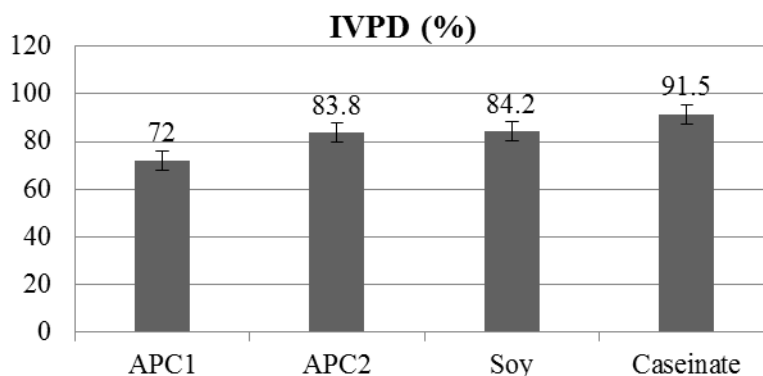
ether extractable content of samples effectively. Protein content of API1 and API2 was 53.2% and 72.5% respectively, significantly different at  $\alpha$  level 5%.

The difference in protein content for API1 and API2 may be attributed to the extraction method used. Results indicated that the enzyme assisted - alkaline extraction - isoelectric precipitation method can improve the protein content of algae protein isolate better than alkaline extraction - isoelectric precipitation process. Using enzyme cellulase to degrade cellulose in the cell wall of algae led to the increase on protein extraction yield and protein purity. Most of the protein in the algae is sodium soluble, therefore using Creston Conc. cellulase enzyme (Genecor) which can work properly at neutral and alkaline pH was really helpful. As shown in the Table 3.1, content of carbohydrate significantly decreased from 15.5% in API1 to 2.15% in API2.

With protein content higher than 50%, it was also suggested that *Chaetomorpha* algae protein powder could be considered as an additional source of plant protein in food products.

### 3.2 In vitro protein digestibility

The *in vitro* protein digestibility (IVPD) assay is useful in screening new food protein and processing method because of their rapidity. The IVPD mimics conditions simulated by the digestive processes occurring in the human gastrointestinal tract through proteolytic enzymes (i.e. pepsin-pancreatin enzyme system or papain system), measuring the percentage of proteins which is hydrolyzed by such enzymes [16].



**Fig.1:** The *in vitro* protein digestibility values (%) of API1, API2, soy isolate and caseinate isolate supplements. Different letters denote difference at 95% of confidence level ( $P < 0.05$ )

A pepsin-pancreatin digest index was devised for a rapid, accurate estimation of protein quality [2]. Under this condition, IVPD of API1 and API2 from *Chaetomorpha* sp. was 72 and 83.8% respectively. As shown in Table 3.1 compared to API1, API2 contained higher protein and lower carbohydrate. Seaweed are known to possess high value of soluble fiber which is a significant inhibitory effect on pepsine activity. Glycosylation is also a mechanism that protect proteins against proteolysis by digestive enzyme. The interaction between polysaccharides and algal proteins or digestive enzyme suggested as the main cause of weak digestibility of algal protein. Therefore, cellulase treatment to remove polysaccharide was a possible way to increase protein



content and protein digestibility in API2. It was also observed that, IVPD of API2 was similar to soy protein isolate ( $p>0.05$ ) and lower (not much) than that of caseinate isolate supplement ( $p<0.05$ ), which were used as reference.

The IVPD values reported on this study were in agreement with studies that evaluated the *in vitro* digestibility of protein isolate from red seaweed *Hypnea japonica* (IVPD 89%) and green seaweed (IVPD of *Ulva lactuca*, *Porphyra tenera* and *Ulva pertusa* were 85, 70% and 67% respectively) [ 6]. Almeida et al. also report that protein from plant source such as soy and algae exhibit lower protein digestibility when compared to casein and whey proteins [2]. This observation may be attributed to the fact that vegetable sources contain antinutritional factors that form a more complex protein structure, which may decrease protein digestibility.

### 3.3 Amino Acid Analysis

Amino acid composition is an important chemical property of proteins, as it determines their nutritional value. Amino acid compositions of algae protein are presented in Table 3.2.

**Table 3.2:** Amino acid compositions of algae protein

Amino acid	Amino acid content (g/100g protein)	Amino acid	Amino acid content (g/100g protein)
Aspartic acid	11.6	Methionine	3.8
Glutamic acid	15.8	Valine	5.1
Serine	5.0	Phenylalanine	5.1
Histidine	1.8	Isoleucine	3.9
Arginine	5.9	Leucine	8.5
Glycine	4.7	Lysine	5.6
Threonine	4.5	Cystine	3.0
Alanine	7.4	Proline	4.8
Tyrosine	4.5		

Aspartic and glutamic acids constituted a large amino acid fraction of algae protein, compared to foods such as soybeans and eggs [12]. With the presence of these amino acids, algae protein was negative electrically charged and soluble at alkaline pH.

As shown in the Table 3.2, protein of *Chaetomorpha* was rich in lysine (5.6 g/100g protein), an essential amino acid important for children feed formulation. The content of lysine in *Chaetomorpha* sp. was higher than that of *Ulva armoricana* (3.5 g/100g protein) and *Ulva*

*pertusa* (4.5 g/100g protein) [4, 6]. Furthermore, algae protein was rich in essential amino acid, especially leucine, valine and phenylalanine. Total amount of essential amino acids was around 40% w/w of total protein. Plant protein is normally very poor in some essential amino acids, such as methionine, threonine and tryptophan [11]. However, there was high content of methionine, threonine found in protein isolate from *Chaetomorpha* algae and as consequence that makes algae protein become good material for food formulation.

### 3.4 Amino acid score (AAS) and Protein digestibility-corrected amino acid score (PDCAAS) of algae protein isolate

AAS and PDCAAS of API1 and API2 were calculated based on the reference value for adults or children 1-2 years old, suggested by the Joint WHO/FAO/UNU Expert Consultation (2007) [18].

The amino acid score (AAS) is based on the relative content of the essential amino acids and the amino acid requirement pattern. Protein from *Chaetomorpha* sp. received high AAS scores (AAS >1) for all of the essential amino acids investigated. As mentioned above, plant protein is normally very poor in methionine and threonine. However, algae protein showed very high AAS (1.7-2.0) for these both amino acids.

The AAS does not consider whether the protein is digestible or not [14]. Therefore, protein digestibility-corrected amino acid score (PDCAAS) is a recognized and approved method for evaluating protein quality taking into account the AAS and the digestibility parameter of the food matrix. This parameter derives from the AAS, and is corrected based on the digestibility assay of the protein [2, 18].

**Table 3.3:** Amino Acid Score of *Chaetomorpha* protein based on amino acid requirement of non-athletic adults

Amino acid	Content (mg/g)	Amino acid requirement in adults (*)	AAS	PDCAAS	
				API 1	API 2
Isoleucine	39	30	1.3	0.9	1.1
Leucine	85	59	1.4	1.0	1.2
Lysine	56	45	1.2	0.9	1.0
Threonine	45	23	2.0	1.4	1.7
Valine	51	39	1.3	0.9	1.1
Histidine	18	15	1.2	0.9	1.0
Methionine	38	22	1.7	1.2	1.4
Phenylalanine	51	38	1.3	0.9	1.1

*\*Recommended values of the Joint WHO/FAO/UNU Expert Consultation (2007)*

Based on the corrected digestibility calculation, it was observed that API2 exhibited greater PDCAAS ( $P < 0.05$ ) than API1. The PDCAAS values of API2 were  $>1.0$  for all amino acid while the PDCAAS values of API1 were  $<1$  for most of amino acid. It was due to the differences on the overall composition of two products and greater *in vitro* protein digestibility observed on API2.

International health agencies established the daily protein intake requirement based on non-athletic individuals as a recommendation for the general population. According to Joint WHO/FAO/UNU Expert Consultation 2007, API2 can be used as good protein supplement for non-athletic adult.

**Table 3.4:** AAS and PDCAAS of *Chaetomorpha* protein based on amino acid requirement of 1-2 year old children

Amino acid	Content (mg/g)	Amino acid requirement in 1-2 year old children (*)	AAS	PDCAAS	
				API1	API2
Isoleucine	39	31	1.3	0.9	1.1
Leucine	85	63	1.3	1.0	1.1
Lysine	56	52	1.1	0.8	0.9
Threonine	45	27	1.7	1.2	1.4
Valine	51	42	1.2	0.9	1.0
Histidine	18	19	0.9	0.7	0.8
Methionine	38	26	1.5	1.1	1.2
Phenylalanine	51	46	1.1	0.8	0.9

*\*Recommended values of the Joint WHO/FAO/UNU Expert Consultation (2007)*

The amino acid requirement of 1-2 year old children is higher than that of adult because children body cannot synthesize some amino acids, they must obtain them from food. Cysteine (or sulphur-containing amino acids), tyrosine (or aromatic amino acids), histidine and arginine are required by infants and growing children and the deficiency in these amino acids may lead to susceptibility to infectious disease [12].

In terms of individual essential amino acid contents, API2 demonstrated both AAS and PDCAAS value of histidine lower than 1.0. According to Joint WHO/FAO/UNU Expert Consultation 2007 recommendation for 1-2 year old children, histidine was regarded as a limiting amino acid in *Chaetomorpha* algae protein.

Phenylalanine is an essential amino acid that should be taken from diet because it acts as a precursor of tyrosine, and together lead to the formation of thyroxine and epinephrine [12]. Lysine is the first limiting amino acid in plant protein. It is naturally occurring amino acid that is needed for optimal growth in infants. It also promotes bone growth in infants as well as stimulates

secretion of gastric juices [15]. API2 demonstrated AAS value >1 but PDCAAS value <1 for phenylalanine and lysine. This observation corroborates the usefulness of PDCAAS to better investigate the amino acidic quality of food products as the food matrix digestibility is considered [14].

In contrast with API2, API1 scored low PDAAS value for almost amino acid. However leucine, threonine and methionine values on API1 were above the reference value for 1-2 year old children. Similar low PDCAAS value of plant protein were reported by Kalpanadevi (2013) [11] and Zhang W. et al. (2010) [16].

## 4 CONCLUSIONS

API2 product from *Chaetomorpha* sp. contained 72.5% w/w protein and exhibited high digestibility (83.8%), similar ( $p>0.05$ ) to soy protein isolate and lower ( $p<0.05$ ) than that of caseinate isolate supplement.

According to the WHO/FAO/UNU protein standard for non-athletic adult, API2 scored high AAS, the PDCAAS values were >1.0. However API2 exhibited suboptimal score values for several EAA and was unable to supply the suggested children EAA requirement.

In addition, studies on the toxicity and *in vivo* digestibility of protein should be conducted to confirm possibility of using algae protein in food products.

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## UTILIZATION OF GAC FRUIT (*MOMORDICA COCHINCHINENSIS* SPRENG.) IN MAKING JAPANESE WAXY RICE CAKE (MOCHI)

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### ABSTRACT

The purpose of this study was to evaluate the potential of using Gac fruit in making mochi. Two types of dried Gac aril (full-fat and defatted) were added in the mochi skin. Meanwhile, the standard filling (red bean sweet paste) was substituted with Gac pulp powder as well as Gac aril oil. All formulations were pre-selected by the sensory evaluation. The quality attributes ( $\beta$ -carotene and lycopene contents, physicochemical characteristics, and sensory properties) of six different Gac mochi were compared with the control mochi which was absent in Gac materials. With increasing amount of Gac incorporation, especially Gac oil, the  $\beta$ -carotene and lycopene content of the Gac mochi greatly increased and were significantly higher than those of the control sample. The physicochemical properties of rice cake were also affected as the addition of Gac oil and Gac full-fat aril led to an increase in the fat content, and substitution with Gac pulp led to decrease in protein content. Sensory quality characteristics of mochi were evaluated including appearance, hardness, chewiness, smoothness, ease-to-eat, and flavor. The best Gac mochi with high levels of  $\beta$ -carotene and lycopene contents and high degree of acceptance was discovered. It was possible to include 5% full-fat Gac aril in the skin, and to include 10% Gac pulp and 3.2% Gac oil in the filling.

**Keywords:** beta-carotene, Gac fruit, lycopene, mochi

### 1 INTRODUCTION

Gac fruit (*Momordica Cochinchinensis* Spreng.) is indigenous to South East Asia and India. Gac fruit comprises orange skin containing spines, yellow pulp (49%, by weight), and aril (red flesh surrounding the seeds). The aril that contains the highest level of carotenoids accounts for only 18% [1]. The brilliant red-orange hue of ripe Gac fruit is due to its high content of  $\beta$ -carotene and lycopene, much higher compared to that in other lycopene-rich fruits and vegetables. Specifically, the content of lycopene in the Gac aril, is approximately 10 times higher than in asparagus, cabbage, carrots, and tomatoes [2]. The Gac yellow pulp has the high amount of lutein [3].

Moreover, the Gac aril also comprises of high levels of fatty acids, ranging from 17% to 22% by weight [4, 5]. Oil extracted from the Gac fruit aril (Gac oil) showed a total carotenoid concentration of 5700 µg/mL, with 2710 µg of that being beta-carotene. This oil also included high levels of vitamin E [4, 6]. The fatty acids in the Gac aril are essential for the absorption of fat-soluble nutrients including carotenoids in a diet typically low in fat [6, 7]. Indeed, Gac oil has been readily accepted by women and children in Vietnam, and consumption of the oil reduced lard intake [7, 4].

In Vietnam, ripe Gac fruits are most commonly used for the preparation of a special food- the Gac aril cooked in glutinous rice, which is usually served at festive occasions such as weddings, the Lunar New Year, as well as used a typical breakfast. Since all parts of the fruit can be processed into nutrient supplements (Gac oil, Gac oil capsule, Gac powder) or Gac natural coloring agents (orange and yellow colorants), it is reasonable to state that Gac fruit has the potential to become a high-value crop. The plant, however, remains underutilized. There is limited information on its requirements in production, and the processing of health products from the fruits is a relatively new area of endeavor. The versatility of the fruit is highlighted through processing options outlined for fruit aril, seeds, pulp and skin into powders or encapsulated oil products [1].

Mochi is the Japanese rice cake made by pounding the pre-soaked and steamed short-grain glutinous rice into a paste and molding it into shapes which can be eaten right away, or cured and dried for later use [8]. One of the most famous mochi in Japanese is Daifuku mochi which consists of a small round mochi stuffed with a sweet filling (commonly, red bean paste). The cake greatly increased in popularity as gifts on ceremonial occasions. As a result, not only being very popular in Japan, Daifuku is also well-known all around the world as a dessert representing Japanese cuisine. In Vietnam, mochi is freshly served in Japanese restaurants and can be found in some supermarkets and Japanese grocery stores. Of central concern, therefore, to enhance the availability and affordability as well as quality in term of nutritional value of the new product, it is worth noticing that Gac fruits, especially the Gac pulp, which is normally discarded during processing, could have been better utilized. This can be primarily attributed to bioactive compounds such as lycopene, β-carotene, and polyphenolics from Gac pulp, Gac aril, and Gac oil. Furthermore, the mochi with the flavor and color of Gac fruit may be readily accepted by the Vietnam customers. The harmony of Gac fruit and glutinous rice, together with red bean paste, makes this fusion of two different cuisines a perfect choice for customers who are seeking a new mochi with a distinctive flavor.

This study was aimed at evaluating the feasibility of Gac mochi product as an option to utilize Gac fruit in Vietnam as well as determining its quality and a consumers' acceptance. The results could be utilized for development of a promisingly new food product - Gac mochi, with additional health benefits.



## 2 MATERIALS AND METHODS

### 2.1 Materials

Gac fruits were purchased from local markets in Ho Chi Minh City. The round-shape Gac fruits with the bright orange skin and no sign of damage were chosen. The fresh Gac pulp was removed, cut into small cubes (1x1 cm) and dried in an oven at 60 °C for 20 h. The dried Gac pulp was then ground into Gac pulp powder, sieved, and kept in the zipper bags in the desiccator for later use. Gac fruit products including Gac oil, full-fat Gac aril powder, and defatted Gac aril powder were purchased from GACVIET Limited Company, kept in sealed aluminum foil bags.

The Vietnamese glutinous rice was chosen instead of using Japanese waxy rice. Glutinous rice flour and corn flour, which was used as the coating flour, were both purchased from Tai Ky Food Flour Corporation. The unpolished red beans were purchased from a local supplier.

All chemicals (grade of AR) used for the analysis of final products were purchased from local agents in Ho Chi Minh City.

### 2.2 Gac mochi skin preparation

There were 2 groups of mochi skins: full-fat (a), and defatted (b). Nine different formulations of each type of Gac mochi skin were made (Table 1), following the brief procedure bellow. Rice flour, water, sugar, and a different proportion of Gac aril powder were mixed well in a microwavable bowl, then cooked in a microwave 3 times (1 min, 1 min, and 30 s) at high power (800 W) until the mixture became homogeneous and almost translucent. The mochi dough (cooked mochi mixture) was set to cool down, then spread into a thin layer, and cut into (1x3 cm) sticks as samples for sensory evaluation. Corn starch was used to prevent sticking.

**Table 1:** Formulation of Gac mochi skins

Type of Powder	Sample Code	Rice Flour	Water	Sugar	Gac Aril Powder
(a) Full-fat	377	50 g	90 mL	3 g	3 g
	213				4 g
	318				5 g
	245			5 g	3 g
	397				4 g
	163				5 g
	764			7 g	3 g
	231				4 g
	958				5 g



Type of Powder	Sample	Rice Flour	Water	Sugar	Gac Aril
(b) Defatted	136	50 g	90 mL	3 g	3 g
	312				4 g
	467				5 g
	592			5 g	3 g
	831				4 g
	295				5 g
	973			7 g	3 g
	245				4 g
	375				5 g

### 2.3 Gac mochi filling preparation

The fillings for Gac mochi, divided into 3 groups: (I) No Gac oil, (II) Less Gac oil, and (III) More Gac oil, were prepared by the procedure as follows. The beans were washed and cooked in a pressure cooker for 1 h. After rinsing all excess water, it was ground into a paste, together with Gac pulp powder and sugar. The mixture was then brought back to heat for 5 min. Then, Gac oil was added and mixed well until the homogenous mixture was achieved. All formulations for Gac mochi fillings are shown in Table 2.

**Table 2:** Formulation of Gac mochi fillings

Type	Sample Code	Red Bean	Water	Gac Aril Oil	Sugar	Gac Pulp Powder
(I) No Gac oil	379	300 g	1000 mL	0 g	230 g	70 g
	256				250 g	50 g
	891				270 g	30 g
(II) Less Gac oil	419	300 g	1000 mL	10 g	230 g	70 g
	306				250 g	50 g
	124				270 g	30 g
(III) More Gac oil	657	300 g	1000 mL	20 g	230 g	70 g
	233				250 g	50 g
	842				270 g	30 g

## **2.4 Development of Gac mochi product**

From the results of rating tests, two kinds of mochi skins and three types of mochi filling which achieved the highest score from 20 panelists, were combined to build Gac mochi products. The control sample which was a mochi prepared in the traditional way as it was not Gac-incorporated.

To make a mochi, the mochi skin (20 g) was firstly spread into a thin layer (2 mm) by the rolling spin, while the mochi filling (10 g) was formed by hands into small balls. The filling was then wrapped by the skin and formed by hands into a ball. Corn starch was used during processing to prevent sticking. Mochi cakes were packaged separately in the refrigerator and kept cold (4-5 °C). For the sensory evaluation, the samples were prepared from the newly-made mochi after few hours chilling in the fridge. The best product's formulation was determined based on the results of sensory evaluation, the nutritional and physicochemical properties analysis.

## **2.5 Determination of $\beta$ -carotene and lycopene content**

The methods of Nagata and Yamashita [9], Bhumsaidon and Chamchong [10] were applied with a slight modification. 5 g of Gac mochi fillings were put in a test tube. The mixed solvents of acetone and hexane in the ratio 4:6 (volume per volume) was then added (10 mL) and mixed well by vortex for 1 min. After several steps for separations and purification, the final solution was transferred and adjusted to a volume of 25 mL. Then, the light absorption values (A) at 453, 505, 663 and 645 nm wavelength were recorded for the determination of the lycopene and  $\beta$ -carotene contents in each sample. The contents of  $\beta$ -carotene and lycopene were calculated based on the equation (1) and (2), respectively.

$$(1) \beta\text{-carotene (mg/100 mL)} = 0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.452A_{453}$$

$$(2) \text{Lycopene (mg/100 mL)} = -0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$$

Where A is the absorbance of the sample at a specific wavelength.

## **2.6 Determination of physicochemical properties**

The moisture, ash, lipid, and protein contents of Gac mochi samples were determined by Association of Official Analytical Chemists [11] methods with slight modifications. All analyses were performed in duplicate. The carbohydrate content was then calculated from the analysis results. The filling and mochi of Gac mochi samples were analyzed separately. Knowing the ratio of a filling to a skin (1: 2), the exact value for each physiochemical properties of Gac mochi sample was calculated.

## **2.7 Sensory evaluation**

*For skins and fillings:*

20 panelists (21 to 29 years old) who have been trained with or familiar with sensory evaluation participated the sensory test. For Gac mochi skin rating test, panelists gave score in 5 attributes,

namely color, smoothness, stickiness, sweetness and overall acceptance, on the 9-point hedonic scale for samples in 2 batches. For Gac mochi filling, samples were presented to panelists in 3 batches and were evaluated in 5 attributes, namely color, flavor, smoothness, sweetness and overall acceptance.

*For Gac mochi samples.*

Each panelist was given a set of 7 mochi samples (randomly labeled with three-digit code). In this descriptive sensory analysis [12], quality characteristics of Gac mochi were appearance, hardness, smoothness, stickiness/adhesiveness, chewiness (elasticity), ease to eat, and flavor. The score for each parameter ranged from 1 to 5 (very bad – bad – satisfied – good – very good). Each parameter was assigned a different factor of significance. The definition and the factor of significance for all parameters are shown in Table 3 [13]. The total score was obtained by summing the scores for each parameter, previously multiplied by the corresponding factor.

**Table 3:** Definitions and corresponding factors of eight parameters for Mochi

<b>Parameter</b>	<b>Definition</b>	<b>Factor of significance (<math>\Sigma=4</math>)</b>
<b>Appearance</b>	The size, shape, color, structure of the sample	0.7
<b>Hardness</b>	The degree of force required to achieve a deformation	0.5
<b>Smoothness</b>	The surface properties of the sample analyzed/felt by tongue and palate	0.7
<b>Stickiness</b>	The extent of sample adhesive on the teeth during/after biting	0.3
<b>Chewiness</b>	Degree to which a deformed sample return to its original shape after removing the deforming force by the molar teeth	0.3
<b>Ease to eat</b>	Physical effort required during total eating processes	0.5
<b>Flavor</b>	The taste and smell of the sample	1

## 2.8 Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD). The results were statistically analyzed using the ANOVA and the Turkey test. Significant level was considered at  $p < 0.05$ .

### 3 RESULTS AND DISCUSSION

#### 3.1 Gac mochi formulation

The average scores in 5 attributes for the mochi skins from different formulas are shown in Table 4. In group (a), where full-fat aril was used, sample 764 obtained the highest mean scores in 3 attributes, namely color, sweetness, and overall acceptability, while the scores in other 2 attributes were quite high. An increase in levels of sugar as well as reduction in the amounts of full-fat Gac aril powder, positively affected the taste, color, and overall acceptability as the samples had the high sweetness, the mild Gac flavor, and the appealing color. In group (b), where defatted aril was used, the Gac flavor was weaker compared to the full-fat one. So, the higher level of defatted Gac aril powder gave a stronger Gac flavor. Furthermore, the Gac aril created intense color for Gac mochi skin which was preferred by the panel. As the result, sample 375 was the most preferable by panelists in group (b). Hence, sample 764 and 375 were chosen as representatives of group (a) and (b), respectively, to develop the Gac mochi products.

**Table 4:** Rating test of color, smoothness, stickiness, sweetness, and overall acceptability of eighteen samples of two types of mochi skin in 9-point scale

	Sample	Color	Smoothness	Stickiness	Sweetness	Overall
(a) Full-fat	377	6,60 ± 0.50	6.75 ± 0.85	6.50 ± 0.83	5.85 ± 1.09	5.80 ± 0.89
	213	6,30 ± 0.73	6.50 ± 0.67	6.15 ± 0.67	6.05 ± 0.89	6.05 ± 0.69
	318	6.55 ± 0.94	6.70 ± 1.00	6.55 ± 1.00	5.80 ± 0.70	5.95 ± 0.83
	245	5.90 ± 0.79	6.30 ± 0.81	5.85 ± 0.81	5.25 ± 0.97	5.35 ± 0.88
	397	6.10 ± 0.79	6.20 ± 1.08	5.70 ± 1.08	5.05 ± 0.89	5.05 ± 1.10
	163	6.30 ± 0.80	6.65 ± 0.89	6.20 ± 0.89	5.20 ± 1.01	5.55 ± 1.05
	764	6.75 ± 0.55	6.60 ± 0.80	6.30 ± 0.80	6.80 ± 0.70	6.45 ± 0.83
	231	5.95 ± 0.89	6.30 ± 0.85	6.25 ± 0.85	5.85 ± 0.88	5.65 ± 0.59
	958	6.55 ± 0.76	6.50 ± 1.10	5.95 ± 1.10	6.25 ± 1.12	5.75 ± 0.97
(b) Defatted	136	5.95 ± 0.50	6.60 ± 0.85	6.30 ± 0.83	4.90 ± 1.09	5.40 ± 0.89
	312	5.50 ± 0.73	4.95 ± 0.76	4.90 ± 0.67	4.60 ± 0.89	4.55 ± 0.69
	467	6.15 ± 0.94	6.05 ± 0.92	5.45 ± 1.00	5.45 ± 0.70	5.15 ± 0.83
	592	6.00 ± 0.79	6.45 ± 0.92	6.40 ± 0.81	5.50 ± 0.97	5.60 ± 0.88
	831	6.45 ± 0.79	6.65 ± 0.89	6.60 ± 1.08	5.75 ± 0.89	5.95 ± 1.10

Sample	Color	Smoothness	Stickiness	Sweetness	Overall
295	6.90 ± 0.80	6.70 ± 0.93	6.50 ± 0.89	5.55 ± 1.01	5.80 ± 1.05
973	6.90 ± 0.49	6.70 ± 0.60	6.50 ± 1.03	5.55 ± 0.99	5.80 ± 0.88
245	6.85 ± 0.89	6.60 ± 0.86	6.55 ± 0.85	6.40 ± 0.88	6.15 ± 0.59
375	7.05 ± 1.05	6.90 ± 0.79	6.95 ± 0.39	7.05 ± 0.83	6.65 ± 0.75

Values reported are means ± standard deviation. Samples with highlight were the most highly preferred.

As for mochi fillings, increase in Gac pulp powder incorporation resulted in increased sense of bitter taste, a loss in sweetness, and the decreasing score for smoothness (Table 5). Also, the mean scores of smell and color decreased as the level of substitution of Gac pulp powder in the mochi fillings increased. The samples with a higher content of sugar and a lower content of Gac pulp powder were more preferred. Hence sample 891, 124, and 842 were selected for further study as a representative for group (I), (II), and (III), respectively. It was also observed that the addition of Gac oil negatively affected the smell of mochi filling as comparing the mean score for the smell of 3 selected samples, which only differed each other by the level of Gac oil incorporated

**Table 5:** Rating test of color, smoothness, stickiness, sweetness, and overall acceptability of nine sample of 3 types of mochi fillings in 9-point scale

	Sample	Color	Smell	Smoothness	Sweetness	Overall
(I) No Gac oil	379	6.20 ± 0.70	5.80 ± 0.77	6.00 ± 0.73	5.20 ± 0.62	5.60 ± 0.68
	256	7.05 ± 0.89	6.65 ± 0.59	6.85 ± 0.81	7.05 ± 0.83	6.95 ± 0.76
	891	7.35 ± 0.88	7.45 ± 0.83	7.50 ± 0.76	7.65 ± 0.75	7.55 ± 0.69
(II) Less Gac oil	419	5.95 ± 0.76	6.20 ± 0.83	5.65 ± 0.75	5.20 ± 0.70	5.50 ± 0.61
	306	6.20 ± 0.62	6.25 ± 0.72	5.70 ± 0.86	5.20 ± 0.95	5.65 ± 0.59
	124	6.80 ± 0.83	6.95 ± 0.76	6.45 ± 0.89	7.15 ± 0.88	6.95 ± 0.69
(III) More Gac oil	657	6.65 ± 0.49	6.60 ± 0.60	6.30 ± 0.80	6.85 ± 0.67	6.45 ± 0.83
	233	6.00 ± 0.92	6.25 ± 0.85	6.15 ± 0.88	5.85 ± 0.88	5.70 ± 0.57
	842	6.85 ± 0.75	6.25 ± 0.72	6.80 ± 0.83	7.35 ± 0.81	7.20 ± 0.70

Values reported are means ± standard deviation. Samples with highlight were the most highly preferred.

As a result from the sensory tests for mochi skins and fillings, two types of mochi skins (full fat-764; defatted- 375) and three types of mochi fillings (no Gac oil-891; Less Gac oil-124; more Gac oil-842) were selected and combined to form six possible formulas for Gac mochi product. The traditional mochi formulation without any Gac ingredient was used as the control sample.

### 3.2 $\beta$ -Carotene and lycopene content

Table 6 showed the values of  $\beta$ -carotene and lycopene in 100 g mochi. The  $\beta$ -carotene and lycopene contents of sample IIIa and IIIb were the highest as they were composed of Gac filling “III”. Those  $\beta$ -carotene contents reached  $0.4270 \pm 0.0081$  mg/100 g mochi, while the lycopene contents peaked  $0.2380 \pm 0.0055$  mg/100 g mochi. With the increase in the level of Gac oil incorporation,  $\beta$ -carotene and lycopene contents in products dramatically increased. Sample III with the Gac fillings at highest level of Gac oil addition showed a peak for  $\beta$ -carotene and lycopene contents.

**Table 6:**  $\beta$ -Carotene and lycopene contents in Gac mochi versus the control mochi (mg/100 g mochi)

Sample	$\beta$ -carotene (mg/100 g mochi)	Lycopene (mg/100 g mochi)
Ia	$0.1474 \pm 0.0073^b$	$0.0873 \pm 0.0043^b$
Ib	$0.1474 \pm 0.0073^b$	$0.0873 \pm 0.0043^b$
IIa	$0.2384 \pm 0.0175^c$	$0.1879 \pm 0.0035^c$
IIb	$0.2384 \pm 0.0175^c$	$0.1879 \pm 0.0035^c$
IIIa	$0.4270 \pm 0.0081^d$	$0.2380 \pm 0.0055^d$
IIIb	$0.4270 \pm 0.0081^d$	$0.2380 \pm 0.0055^d$
Control	$0.0324 \pm 0.0014^a$	$0.0481 \pm 0.0013^a$

Values reported are means  $\pm$  standard deviation. Means with different letters differed significantly ( $p < 0.05$ )

### 3.3 Nutritional values

Except for the moisture content, the physicochemical properties of Gac mochi products were determined and calculated on a dry basis (Table 7).

The moisture content varied from 52.74% to 54.54%. However, there was no statistically significant difference among samples in moisture content. The ash content ranged from 0.81% to 0.83% was not significantly different ( $P > 0.05$ ) among six samples, while the ash content in the control sample was lower. The protein content was depending on the level of inclusion of Gac aril in skin and, in particularly, Gac oil in mochi fillings. The samples with no Gac oil addition which meant the higher proportion of a protein-dense red bean paste, achieved the highest protein

content. In contrast, with an increase in Gac oil incorporation from 0 g (no Gac oil) to 20 g (more Gac oil), the fat content showed the significant difference, ranging from 0.44 to 1.68% (d.b). Samples made of more Gac oil fillings have higher fat content. Moreover, the type of Gac aril used in the skin also affected the fat content of mochi sample as the full-fat sample showed a higher amount of fat versus that of the defatted sample.

The control sample showed the higher content in protein and the substantially lower content of fat.

**Table 7:** Chemical compositions of Gac mochi products

Sample	Moisture (%wb)	Ash (%db)	Protein (%db)	Fat (%db)	Carbohydrate (%db)
<b>Ia</b>	53.11 ± 0.68 <sup>a</sup>	0.81 ± 0.00 <sup>a</sup>	8.69 ± 0.02 <sup>d</sup>	0.68 ± 0.00 <sup>b</sup>	89.82 ± 0.01 <sup>c</sup>
<b>Ib</b>	54.54 ± 0.58 <sup>a</sup>	0.81 ± 0.00 <sup>a</sup>	8.61 ± 0.01 <sup>c</sup>	0.44 ± 0.00 <sup>a</sup>	90.14 ± 0.01 <sup>f</sup>
<b>IIa</b>	52.95 ± 0.66 <sup>a</sup>	0.82 ± 0.01 <sup>a</sup>	8.64 ± 0.00 <sup>c</sup>	1.24 ± 0.00 <sup>d</sup>	89.31 ± 0.01 <sup>c</sup>
<b>IIb</b>	54.37 ± 0.56 <sup>a</sup>	0.81 ± 0.01 <sup>a</sup>	8.56 ± 0.01 <sup>b</sup>	1.00 ± 0.00 <sup>c</sup>	89.63 ± 0.02 <sup>d</sup>
<b>IIIa</b>	52.74 ± 0.71 <sup>a</sup>	0.83 ± 0.01 <sup>a</sup>	8.56 ± 0.01 <sup>b</sup>	1.68 ± 0.00 <sup>f</sup>	88.94 ± 0.00 <sup>a</sup>
<b>IIIb</b>	54.17 ± 0.61 <sup>a</sup>	0.83 ± 0.01 <sup>a</sup>	8.48 ± 0.00 <sup>a</sup>	1.44 ± 0.00 <sup>c</sup>	89.25 ± 0.00 <sup>b</sup>
<b>Control</b>	54.06	0.76	9.95	0.46	

Values are means ± standard deviation, reported on dry basis, except the MC. Means with different letters differed significantly ( $p < 0.05$ )

### 3.4 Sensory evaluation

The results from sensory test for seven different attributes, including appearance, hardness, smoothness, stickiness, chewiness, ease to eat, and flavor are presented in Table 8.

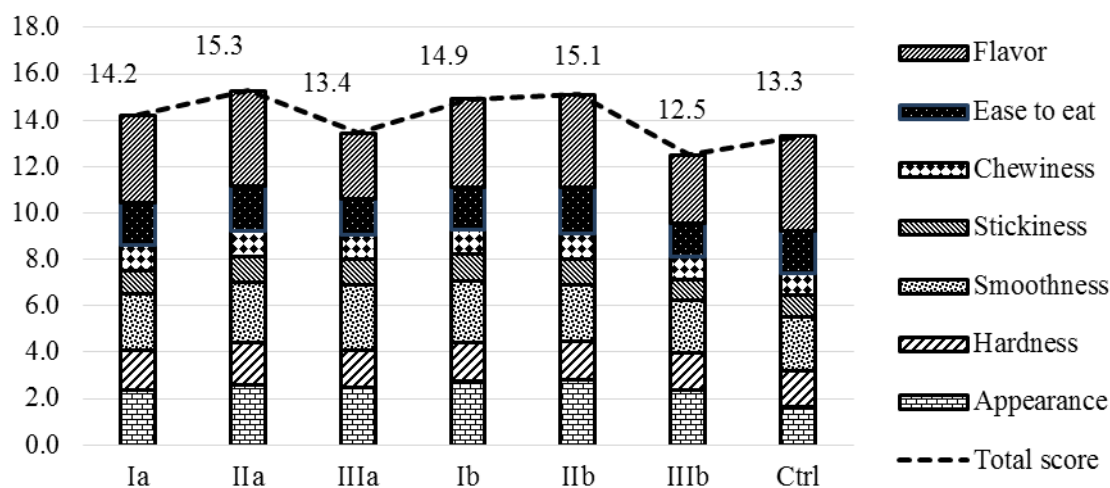
**Table 8:** Descriptive sensory evaluation of seven attributes: appearance, hardness, smoothness, stickiness, ease-to-eat, and flavor of six Gac mochi products and a control mochi in 5-point scale

Sample	Appearance	Hardness	Smoothness	Stickiness	Chewiness	Ease to eat	Flavor
<b>Ia</b>	3.40 ± 0.50 <sup>b</sup>	3.40 ± 0.60 <sup>ab</sup>	3.50 ± 0.51 <sup>ab</sup>	3.30 ± 0.57 <sup>abc</sup>	3.70 ± 0.57 <sup>bc</sup>	3.60 ± 0.50 <sup>bc</sup>	3.80 ± 0.62 <sup>b</sup>
<b>IIa</b>	3.70 ± 0.47 <sup>bc</sup>	3.70 ± 0.47 <sup>b</sup>	3.70 ± 0.57 <sup>abc</sup>	3.70 ± 0.57 <sup>cd</sup>	3.60 ± 0.59 <sup>abc</sup>	3.90 ± 0.31 <sup>c</sup>	4.10 ± 0.45 <sup>b</sup>
<b>IIIa</b>	3.55 ± 0.51 <sup>bc</sup>	3.25 ± 0.44 <sup>a</sup>	4.00 ± 0.56 <sup>c</sup>	3.60 ± 0.50 <sup>bcd</sup>	3.50 ± 0.51 <sup>abc</sup>	3.20 ± 0.41 <sup>ab</sup>	2.80 ± 0.52 <sup>a</sup>
<b>Ib</b>	3.90 ± 0.45 <sup>c</sup>	3.40 ± 0.50 <sup>ab</sup>	3.80 ± 0.52 <sup>bc</sup>	3.80 ± 0.41 <sup>d</sup>	3.45 ± 0.51 <sup>abc</sup>	3.70 ± 0.47 <sup>c</sup>	3.80 ± 0.70 <sup>b</sup>

Sample	Appearance	Hardness	Smoothness	Stickiness	Chewiness	Ease to eat	Flavor
<b>IIb</b>	4.00 ± 0.46 <sup>c</sup>	3.35 ± 0.49 <sup>ab</sup>	3.50 ± 0.51 <sup>ab</sup>	3.65 ± 0.59 <sup>cd</sup>	3.75 ± 0.44 <sup>c</sup>	3.90 ± 0.31 <sup>c</sup>	4.00 ± 0.46 <sup>b</sup>
<b>IIIb</b>	3.40 ± 0.60 <sup>b</sup>	3.15 ± 0.37 <sup>a</sup>	3.25 ± 0.44 <sup>a</sup>	3.00 ± 0.46 <sup>a</sup>	3.25 ± 0.44 <sup>ab</sup>	2.90 ± 0.45 <sup>a</sup>	2.95 ± 0.51 <sup>a</sup>
<b>Control</b>	2.35 ± 0.49 <sup>a</sup>	3.15 ± 0.37 <sup>a</sup>	3.25 ± 0.44 <sup>a</sup>	3.15 ± 0.37 <sup>ab</sup>	3.20 ± 0.41 <sup>a</sup>	3.70 ± 0.47 <sup>c</sup>	4.05 ± 0.51 <sup>b</sup>

Values reported are means ± standard deviation. Means with different letters differed significantly ( $p < 0.05$ )

For appearance, the mean scores varied from 3.40 to 4.00, which were rated as “satisfied” to “good” and significantly different from the control sample, which was rated as “bad”. The addition of Gac aril powder to the mochi skin created an appealing light red color which received relatively good scores from panelists. For hardness, the scores ranged from 3.15 to 3.70, showing no significant difference among samples and the control sample except for the sample IIa, which achieved the highest score. With respect to smoothness, sample IIIa was rated as “good” while other samples including the control sample achieved a “satisfied” score. Though there was a statistically significant difference among seven samples in stickiness and chewiness, all presented a “satisfied” result. The perfect mochi has the perfect balance between smoothness and stickiness so that it is not inextensible and fragile but rather extensible yet firm [14]. Meanwhile, the ease-to-eat is truly worth a concern as there were some accidents involving choking on mochi, especially in the elderly [15]. Only sample IIIb was rated as “bad”, while other samples were rated as easy to eat. The scores of flavor showed a wide range from 2.8 to 4.10. Sample IIa received highest score in flavor, followed by the control sample and sample IIb.



**Figure 1:** Total sensory score of Gac mochi samples and the control sample

The total sensory score was obtained by summing the scores for each parameter, previously multiplied by the corresponding factor (Fig. 1). The total sensory score ranged from 12.5 to 15.3. The highest scores were given to the mochi cakes supplemented with a medium amount of Gac



oil in the mochi filling, of which the best was sample IIa. It also presented the highest score in 'Hardness', 'Stickiness', and 'Flavor'. With only a gap of 0.2 in total score, the sample IIb was at the second place. In addition, it took the first place at three attributes which are 'Appearance', 'Chewiness', and 'Ease-to-eat'. The control sample achieved the total score of 13.3, with lowest score in 'Appearance'. Meanwhile, sample IIIb containing Gac defatted aril in the skin and the highest levels of Gac oil in the filling, got lowest total score (12.5), as it did not receive preferences in 'Ease-to-eat' and 'Flavor'.

## **4 CONCLUSIONS**

The feasibility of supplement of Gac oil, Gac aril, and Gac pulp in mochi was successfully evaluated. With incorporation of the Gac ingredients, especially the Gac oil, the  $\beta$ -carotene and lycopene contents in mochi was significantly increased. Gac proportion in the product formulation influenced the physicochemical properties as the Gac mochi contains higher content of fat, slightly lower content of protein compared to the traditional one. Sensory characteristics were also affected, especially the appearance which was considerably enhanced.

The best formulation was found as the sample IIa. The product made by this combination achieved high acceptability. Tracing back to Table 6 and Table 1,2 for its formulation, it was possible to include 5% full-fat Gac aril in the skin, and to include 10% Gac pulp and 3.2% Gac oil in the filling. The product made by this formula contained high levels of  $\beta$ -carotene and lycopene (0.2384 mg and 0.1879 mg per 100 g mochi, respectively).

Well-known for its enormous health benefits and abundance in Vietnam, Gac fruit potentially becomes a high-value crop. The 'fruit from heaven', however, is still underutilized. By using Gac fruit in making mochi, the nutritional, bioactive and sensory characteristics of mochi was considerably improved. Therefore, it could be a promising option to utilize the fruit as well as fetch a good economic value. Further investigation is necessary to study the economic aspects of the products before recommending for a commercial level.

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## **SESSION 2**

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# **FOOD CHEMISTRY, MICROBIOLOGY, QUALITY AND SAFETY**



## ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF EXTRACT FROM SOME GREEN VEGETABLE LEAVES IN VIETNAM AND ITS APPLICATION IN FOOD PRODUCT

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### ABSTRACT

Green leaves such as: Chive (*Allium tuberosum* Rottler ex Spreng), crown-daisy (*Chrysanthemum coronarium*), perilla (*Perilla frutescens*) and guava (*Psidium guajava*) are some common vegetables which have rich in phenolic compounds and have a wide range of biological functions such as: antioxidant and antimicrobial activities. The research aimed to investigate these bioactive activities in extraction and also focus in the application in food product. The antioxidant activity, total phenolic content (TPC) were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric-reducing antioxidant power (FRAP). The assays of antimicrobial activity were performed by using agar well diffusion method against standard of *Samonella typhimurium* (ST), *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA), *Bacillus cereus* (BC), *Aspergillus niger* (AN), *Trichoderma longibrachium* (TT) and *Fusarium moniliforme* (FM). The antioxidant and antimicrobial effects of guava leaf extract on marinated chicken wing during storage for 10 days at 2 – 4°C were studied. Chicken wing was treated with BHT (possitive control for antioxidant experiment), Potassium sorbate (possitive control for antimicrobial experiment), guava leaf extract (1% and 0.5% v/w) and the results were compared to these obtained for chicken wing without any additive (control). The extraction of guava leaf showed the highest antioxidant with TPC, IC<sub>50</sub> and FRAP in comparision to that of others. In addition, guava leaf extract also the one that had antimicrobial activity in this studied. Moreover, chive extract was the only one showing antifugal activity in comparision to those of leafy green extracts. The pH, TBARS, TVC and *Staphylococcus aureus* of of chicken wing treated with 1% extraction samples were lower than control sample.

**Keywords:** antibacterial activity; antioxidant activity; Chive (*Allium tuberosum* Rottler ex Spreng), crown-daisy (*Chrysanthemum coronarium*), perilla (*Perilla frutescens*) and guava (*Psidium guajava*).

## 1 INTRODUCTION

In recent years, consumers are more interested in industrial products that do not use synthetic additives, natural-friendly products, and less processed. Antioxidants, antimicrobial agents have been widely used because of its convenient, high efficiency during application and storage. However, the health effects of these synthetic substances have left some potential symptoms and still a matter of concern. Since then, the demand for natural antioxidants and antimicrobials has been increasing, and studies on the absorption and application of antioxidants and antibiotics from natural materials have also increased.

We have known how to harness the abundant source of vegetation on Earth for many different kind of purposes in a long time. Plants not only provide oxygen to humans, regulate the climate through photosynthesis, but also play an important role in the supply of food, essential oils, wood products, etc. In the course of cultivation, people have learned and improved the cultivation process to obtain more and more valuable products from plants such as: leaves, fruit, plants, tubers - roots, nut, etc. In general, all parts of plants can be exploited by humans. Green leafy egetables are widely consumed in Asia and are prepared by many different cooking methods. These vegetables have been eaten for centuries and are classified as Generally Recognized As Safety (GRAS). In particular, these foods contain large amounts of polyphenols (such as: phenolic acids, flavonoids and aromatic compounds) which are the richest phytochemicals in the human diet (Faller & Fialho, 2009).

Meat products are usually sold in refrigerators at temperatures between 2 and 4°C. They are very easily spoiled during storage by two main causes: microorganism growth and oxidation (Sebranek *et al*, 2005). Lipid oxidation and microbial growth during storage can be reduced by applying antioxidant and antimicrobial agents to the meat products, leading to a retardation of spoilage, extension of shelf-life, and maintenance of quality and safety (Devatkal & Naveena, 2010).

Extraction from leaves can be considered as natural sources of antioxidants and antimicrobial substances, the addition of ingredients to food ingredients is considered safe and human-friendly. Nowaday, studies on green leafy vegetables that are commonly used in daily life but the examining and comparing of their oxidative and antimicrobial activities with these of other plants still have been quiet limited. Therefore, we will investigate the antioxidant activity, antimicrobial activity of chive (*Allium tuberosum* Rottler ex Spreng), perilla (*Perilla frutescens*), crown-daisy (*Chrysanthemum coronarium*) and guava leaves (*Psidium guajava*) and their application in product preservation to replace synthetic antioxidants and antimicrobial agents.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Fresh leaves of Chive (*Allium tuberosum* Rottler ex Spreng), crown-daisy (*Chrysanthemum coronarium*), perilla (*Perilla frutescens*) and guava (*Psidium guajava*) were purchased from local farms in Long An province, Southern of Vietnam. The plant stems from all plants were removed, and the leaves were washed and dehydrated in a dry oven at 45<sup>0</sup>C for 48h. The dried plants were then finely pulverized using an electric grinder, sieved, and stored at -18<sup>0</sup>C until experimented.

### 2.2 Chemicals and reagents

DPPH, Trolox, Folin-Ciocalteu, Gallic acid, BHT, Potassium sorbate and TBA (thiobarbituric acid) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol were obtained from Xilong (Shantou, Guangdong, China).

### 2.3 Preparation of leaf extract

Fifty grams of each vegetable powder were soaked in 400 mL of 70% (v/v) ethanol for 6 hours at 60<sup>0</sup>C using an constant shaking (100 rpm). After filtration with Whatman No. 2 filter paper, the obtained extract was concentrated under reduced pressure in a water bath set at 50<sup>0</sup>C using a rotary evaporator (R-100, Büchi, Fawil, Switzerland) and the extracts were freeze dried. Dried extracts were placed in sealed bottles and stored at -18<sup>0</sup>C before use. The extracts were dissolved in 95% ethanol for analysis of antioxidant and were dissolved in distilled water (1%, w/v) for antimicrobial properties and application on chicken meat products. The residual was weighed, and the extraction yield of each plant material was calculated.

### 2.4 Analysis of leaf extract

#### 2.4.1 Determination of total phenolic content

The content of total phenolics was measured spectrophotometrically using the Folin–Ciocalteu colorimetric method (Dewanto *et al.*, 2002). All plant extracts were diluted with extraction solvent (70% ethanol or distilled–deionized water) to obtain readings within the standard curve range of 0.0 - 0.8 mg gallic acid/mL. Briefly, 100 µL of diluted plant extract or gallic acid standard solution was mixed well with 2 mL of 2% (w/v) Na<sub>2</sub>CO<sub>3</sub> solution. The mixture was then left to stand for 3 min, after which 100 µL of Folin–Ciocalteu reagent was added. After letting the mixture sit for 30 min at room temperature for color development, the absorbance was measured at 750 nm using a UV–visible spectrophotometer. Results are expressed as mg gallic acid equivalent (GAE)/g dried plant.

#### 2.4.2 DPPH radical scavenging activity

The antioxidant activity of the DPPH was determined based on the reaction of antioxidants found in the DPPH radical scavenging test. DPPH is a nitrogen-free radical, stable, violet color.

Maximum DPPH uptake in ethanol or methanol was recorded at 517nm or 515nm (Blois, 1958, Brand-Williams, Cuvelier *et al.*, 1995). When antioxidants are added to the DPPH solution, the antioxidants will change the DPPH base to become light yellow, thus reducing the DPPH uptake.

The DPPH antioxidant activity is expressed as the IC<sub>50</sub> value - the amount of antioxidant needed to reduce DPPH's initial concentration by 50 percent. The initial DPPH concentration used in this study was  $6.10^{-5}$  M in methanol, the reaction time was 30 minutes, the absorption at 515 nm (Brand-Williams, Cuvelier *et al.*, 1995). The IC<sub>50</sub> value was obtained by constructing a linear line between the sample and the percentage of free radical scavenging, thereby obtaining a value representing the antioxidant content at which a 50 percent reduction in free radical concentration was obtained. The IC<sub>50</sub> value is expressed in units of mg/ml.

#### 2.4.3 Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was performed based on the procedure described by Benzie and Strain (1996) with slight modifications. In this assay, 100 µL of the diluted sample were added to 3 mL of the FRAP reagent and the reaction was monitored after 4 min at 593 nm. The results were expressed as µmol Fe(II)/g per dried weight of vegetable.

#### 2.4.4 Antimicrobial of leaf extract

##### 2.4.4.1 Microorganism strains and culture condition

The reference bacteria strains: *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 9027), purchased from Institute of Food Industry (Hanoi, Vietnam), were used for antibacteria assays. These include two strains of Gram-negative bacteria and two strains of Gram-positive bacteria. All strains of these bacteria are well-known food-borne bacterial pathogens. Before using, the bacterial strains were consecutively subcultured in Tryptic soy broth (TSB) at least three times with 24 h intervals. The 10 ml of culture was transferred to a 10 ml liquid medium, and the inoculated media were incubated at 37<sup>0</sup>C for 24 h.

The reference fungi strains: *Aspergillus niger* (ATCC 16888), *Fusarium moniliforme* (ATCC 38159) and *Trichoderma longibrachiatum* (ATCC 18648), purchased from Institute of Food Industry (Hanoi, Vietnam), were used for antifungal assays. The microorganisms were grown on Potato dextrose agar (PDA) plate at 37<sup>0</sup>C for 48 h. Conidia were harvested by centrifugation at 1000g for 25 min and washed with 10 ml of sterile distilled water. This step was repeated three times. The spore suspension was stored in sterile distilled water at 4<sup>0</sup>C until used.

Culture environment for microorganisms such as: Tryptic Soy Broth (TSB), Muller Hinton Agar (MHA), Potato Dextrose Agar (PDA), Plate Count Agar (PCA), Man, Rogosa and Sharpe (MRS), Baird Parker Agar (BPA) were purchased from Himedia - (Mumbai, India).



#### 2.4.4.2 Antibacterial and antifungal activity of leaf extract

The inhibitory effects of the vegetable extracts on test bacteria and fungi were firstly determined by the agar well diffusion method (Chollet *et al.*, 2008) with some modifications.

For antibacterial assays, the autoclaved media (MHA) were cooled to 50<sup>0</sup>C in a water bath, and an overnight culture of the bacterial strain was added. Pouring 18 ml of this suspension into a sterile Petri dish (90 mm diameter) and dried for at least 1 h at room temperature to reach solidification. 3 wells (6 mm diameter) per Petri dish were cut using a stainless pipe that was custom made for this experiment. Plant extracts were diluted to 50 mg/ml with distilled water, and 50 ml of diluted extract was dispensed into each agar well. The negative control agar well was 0.1 mL of distilled water while the positive control was 0.1 mL Chloramphenicol 50 mg/mL. Plates were stored for at least 1 h at room temperature to allow the extracts to diffuse into the agar before incubation at 37<sup>0</sup>C for 24 h. The inhibitory effect was assessed by measuring the disc diameter of the inhibition zone (clear zone) around the extract using a calliper.

For antifungal assays, the autoclaved media (PDA) were cooled to 50<sup>0</sup>C in a water bath. Pouring 18 ml of PDA into a sterile Petri dish (90 mm diameter) and dried for at least 1 h at room temperature to reach solidification. Spore suspension of the fungi strain was added on the surface of the agar and well spreaded to fully cover. 3 wells (6 mm diameter) per Petri dish were cut using a stainless pipe that was custom made for this experiment. Plant extracts were diluted to 50 mg/ml with distilled water, and 50 ml of diluted extract was dispensed into each agar well. The negative control agar well was 0.1 mL of distilled water while the positive control was 0.1 mL Ketoconazole 50 mg/mL. Plates were stored for at least 1 h at room temperature to allow the extracts to diffuse into the agar before incubation at 37<sup>0</sup>C for 48 h. The inhibitory effect was assessed by measuring the disc diameter of the inhibition zone (clear zone) around the extract using a calliper.

#### 2.4.5 Sample preparation

Chicken wing (61.5 g/100 g moisture, 16.5 g/100 g protein, 9.6 g/100 g fat content) were provided by a supermarket. They were put into a foam box with ice and transferred to the laboratory within 15 min. Chicken wing were then washed, trimmed, par-steamed, marinated and stored at 2 – 4<sup>0</sup>C. The samples were assigned to one of 3 treatments: C: control samples; BHT: positive control with 0.02% BHT; PG-1: treatment with guava leaf extract 1% (v/w) and PG-0.5: treatment with guava leaf extract 1%. Meat samples were aerobically packed in a plastic box and stored at 2 - 4<sup>0</sup>C for 10 days and analyzed for pH, instrumental colour attribute, microbial counts, thiobarbituric acid reactive substances (TBARS), acid value (AV) and peroxide value (PoV). The above described experiment was carried out in triplicate.

#### 2.4.6 Analysis of chicken wing samples

##### 2.4.6.1 pH determination

pH levels were determined according to AOAC (1995). Specifically, a 10.0 g sample of the meat muscle was homogenized in 100 mL distilled water, and the mixture was filtered. The pH of the filtrate was measured using a pH meter (Mettler Toledo 320-S)

##### 2.4.6.2 Color value

The colour of the raw chicken breast meat fillets was determined using a Colour Difference Meter (WSC-S, Shanghai Physics and Optics Instrument Co.). Colour was described in terms of the L\* (lightness), a\* (redness), and b\* (yellowness) colour space values. Measurements were made perpendicular to the fillet surface at five different locations per sample; mean values (L\*, a\*, and b\*) from the samples were analyzed, and triplicate fillets were analyzed to obtain an average colourimetric value.

##### 2.4.6.3 TBARS value

The TBARS value was determined according to Erkan and Özden, with some modifications. Approximately 5.0 g of meat was homogenized with 25 mL 7.5% (w/v) trichloroacetic acid (containing 0.1% EDTA) at 15,000 rev per minute. The mixture was centrifuged at 3600g for 20 min at room temperature. The supernatant (5 mL) was mixed with 5 mL 0.02 mol/L TBA reagent. The mixture was heated in a boiling water bath for 30 min and cooled to room temperature. The absorbance of the resulting supernatant solution was measured using a UV spectrometer (Shimadzu, Japan) at 532 nm against a blank prepared with 5 mL distilled water and 5 mL TBA solution. The amount of TBARS was expressed as mg of malondialdehyde (MDA) per kg meat sample

Samples were submitted to microbial analysis immediately after inoculation and again after 2, 4, 6, 8, and 10 days of refrigerated storage. The following groups of microflora were examined: total viable counts (TVC), lactic acid bacteria (LAB), and *S.aureus* coagulase positive. Total viable counts (TVC) were determined using Plate Count Agar (PCA) after incubation for 48 h at 37°C. Lactic acid bacteria were determined on de Man, Rogosa, and Sharpe (MRS) medium after 72 h incubation at 30°C. *S.aureus* coagulase positive were determined using Baird-Packer agar (BPA) as a medium after 24 h of incubation at 30°C.

#### 2.4.7 Analytical methods

All the experiments were performed in triplicate, and the results were expressed as mean  $\pm$  SD (standard deviation). Statistical analysis was performed using SPSS 13.0 and Excel 2016. Statistical significance was identified at the 95% confidence level ( $P \leq 0.05$ ).

### 3 RESULTS AND DISCUSSION

#### 3.1 Total phenolic content

The total phenolic content of chive, perilla, crown-daisy and guava leaves are shown in Table 1.

**Table 1:** The total phenolic content of chive, perilla, crown-daisy and guava leaves

Leaf	Total phenolic content (mg GAE/g)
Chive	29.05 ± 0.27 <sup>b</sup>
Perilla	21.56 ± 1.08 <sup>c</sup>
Crown-daisy	18.83 ± 0.34 <sup>c</sup>
Guava	374.63 ± 0.52 <sup>a</sup>

Results are expressed as mean ± SD.

a-c Values with different characters in the same column differ significantly ( $P \leq 0.05$ ).

The results from Table 4.1 show that the total phenolic content of guava leaf was the highest value ( $P \leq 0.05$ ), reaching at 374.63 mgGAE/g dry matter. Following is chive leaf with 29.05 mgGAE/g dry matter. Perilla and crown-daisy had the lowest total phenolic content ( $P \leq 0.05$ ), respectively 21.56 mgGAE/g dry matter and 18.83 mgGAE/g dry matter.

In general, phenolic content in chives, perilla leaves, crown-daisy and guava leaves were different in many scientific publications. This can be explained by differences in the origin of the material, the type of solvent and the condition of the extraction process.

#### 3.2 DPPH and FRAP assays

The results of various antioxidant tests for four kinds of leaf extracts are expressed in Table 2. Because natural antioxidants are often multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to foods. Inconsistent results have been obtained because non-specific one-dimensional methods were used to evaluate natural antioxidant activities (Frankel & Meyer, 2000). Therefore, it is necessary to perform more than one type of antioxidant capacity measurement to take into account the various mechanisms of antioxidant action.

Table 2 shows that the IC<sub>50</sub> of crown-daisy was the highest value (20.07 mg/ml) ( $P \leq 0.05$ ) while that of guava leaf was the lowest one (0.35 mg/ml). By contrast, the FRAP value of guava leaf was the highest (3505.87  $\mu\text{mol Fe}^{2+}/\text{g}$ ) while that of crown-daisy was the lowest one (89.05  $\mu\text{mol Fe}^{2+}/\text{g}$ ). The FRAP values were in the range of 89.05 – 3505.87  $\mu\text{mol Fe}^{2+}/\text{g}$ . The vegetables with the highest total FRAP values, in decreasing order, were guava leaf > chives > perilla leaf >

crown-daisy, with total FRAP values of 3505.87, 326.08, 174.20 and 89.05  $\mu\text{mol Fe}^{2+}/\text{g}$ , respectively.

The results showed that there were some variations for antioxidant capacities among different samples. This is also the reason that the results reported by different authors were very different (Isabelle *et al.*, 2010).

**Table 2:** DPPH (IC<sub>50</sub>) and FRAP ability for extracts of chive, perilla, crown-daisy and guava leaves

Leaf	DPPH – IC <sub>50</sub> (mg/ml)	FRAP ( $\mu\text{mol Fe}^{2+}/\text{g}$ )
Chive	4.21 $\pm$ 0.04 <sup>b</sup>	326.08 $\pm$ 1.91 <sup>b</sup>
Perilla	2.34 $\pm$ 0.11 <sup>b</sup>	174.20 $\pm$ 1.21 <sup>c</sup>
Crown-daisy	20.07 $\pm$ 0.18 <sup>a</sup>	89.05 $\pm$ 1.11 <sup>d</sup>
Guava	0.35 $\pm$ 0.04 <sup>c</sup>	3505. 87 $\pm$ 4.34 <sup>a</sup>

Results are expressed as mean  $\pm$  SD.

a-c Values with different characters in the same column differ significantly ( $P \leq 0.05$ ).

### 3.3 Antimicrobial of leaf extract

#### 3.3.1 Antibacterial of leaf extract

The study used the agar well diffusion method with two Gram-negative strains [*Salmonella typhimurium* (ST) and *Pseudomonas aeruginosa* (PA)] and two Gram-positive strains [*Staphylococcus aureus* (SA) and *Bacillus cereus* (BC)]. The positive control was Chloramphenicol 50 mg/ml. Results of the antibacterial activity of chives, perilla, crown-daisy and guava leaf extracts are shown in Table 3.

**Table 3:** Antibacterial activity of leaves extract against microbial strains by agar well diffusion methods

	Inhibited zone (mm)			
	SA (G <sup>+</sup> )	ST (G <sup>-</sup> )	BC (G <sup>+</sup> )	PA (G <sup>-</sup> )
<b>Chloramphenicol (50 mg/ml)</b>	43.68 $\pm$ 1.2 <sup>Aa</sup>	43.73 $\pm$ 1.4 <sup>Aa</sup>	43.56 $\pm$ 0.6 <sup>Aa</sup>	48.62 $\pm$ 1.2 <sup>Ab</sup>
<b>Chives</b>	-	-	-	-
<b>Perilla</b>	-	-	-	-
<b>Crown-daisy</b>	-	-	-	-
<b>Guava</b>	16.22 $\pm$ 1.7 <sup>Bb</sup>	16.12 $\pm$ 1.3 <sup>Bb</sup>	13.28 $\pm$ 1.2 <sup>Ba</sup>	16.46 $\pm$ 0.5 <sup>Bb</sup>

The extract concentration was 50 mg/ml.

The diameter of the inhibited zone is expressed as mean  $\pm$  SD (mm).

Symbol: " - " indicates extracts not resistant to microorganisms at concentrations above 50 mg/ml.

A-B: Different letters in the same column represent significant differences ( $P \leq 0.05$ ).

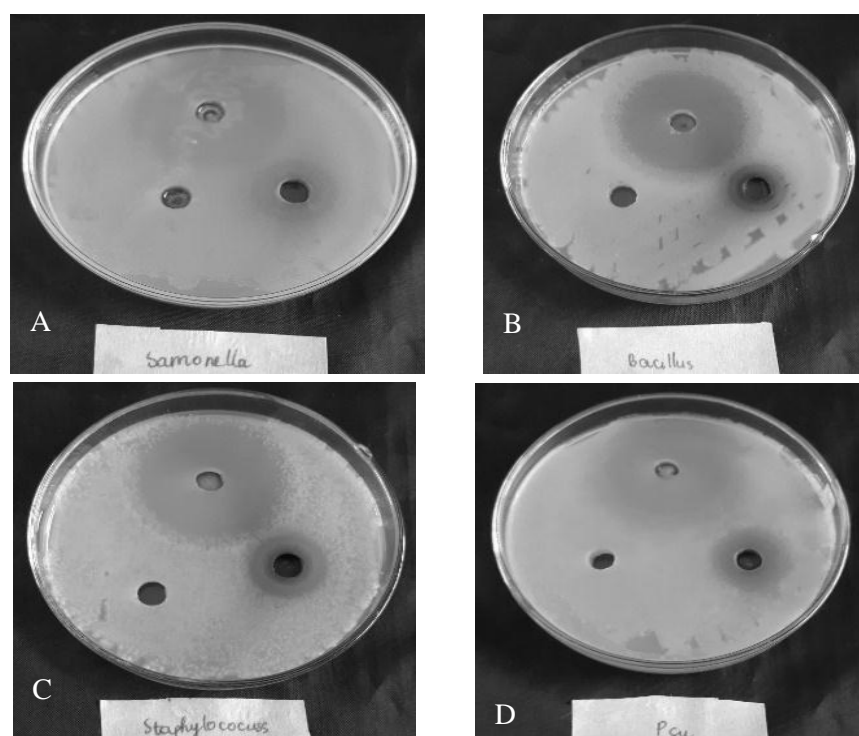
a-b: different letters in the same row represent significant differences ( $P \leq 0.05$ ).

The results from Table 3 showed that only extract from guava leaf had the antimicrobial activity against microorganisms. This can be explained by the fact that in the extraction of chive, perilla and crown-daisy contain phenolic compounds which do not have antimicrobial activity (Du *et al.*, 2011).

According to this test, Gram negative bacteria were more strongly suppressed than Gram-positive bacteria ( $P \leq 0.05$ ). The largest inhibited zone was *Pseudomonas aeruginosa* (16.46 mm) and the smallest one was *Bacillus cereus* (13.28 mm). This suggests that extracts extracted from guava leaves have a more effective with Gram-negative bacteria than that of Gram-positive. This finding is consistent with Fathy M. Soliman *et al.* (2016).

For *Staphylococcus aureus* (Gram-positive), the extract from the guava leaves of the study had an inhibited diameter of 16.22 mm, similar to that of Fathy M. Soliman *et al.* (2016) (the bacterium was 16 mm). Tested for *Bacillus cereus*, the extract from guava leaves was 13.28 mm in diameter. The study by Fathy M. Soliman *et al.* (2016) on *Bacillus subtilis* also had a similarly nearly identical 13 mm of inhibited zone. In antibacterial experiments with *Pseudomonas aeruginosa* (Gram-negative bacteria), the extract from guava leaves in the study had an inhibited zone about 16.46 mm while research by Fathy M. Soliman *et al.* (2016) was 13 mm. This difference can be explained by the difference in the origin of the material, the conditions of extraction and the conditions of the experiment.

The mechanism for the antibacterial activity of leaf green extract is unclear now. However, cell membrane destruction by phenolic compounds and metal complexation with flavonoids have been investigated to clarify the problem of microbial growth. The antimicrobial activity of phenolic compounds can vary from one mechanism to some mechanisms. Phenolic compounds, for example, can break down the cell wall, causing cytoplasmic leakage, altering the composition of fatty acids and phospholipids, affecting the replication of DNA and RNA from there to inhibit the process of protein synthesis. (Du *et al.*, 2011).



**Figure 1:** Antibacterial experiment of the extract from guava leaves

- A. Experiment with *Pseudomonas aeruginosa*      B. Experiment with *Staphylococcus aureus*  
C. Experiment with *Bacillus cereus*      D. Experiment with *Salmonella typhimurium*

### 3.3.2 Antifungal of leaf extract

The agar well diffusion method was used in three cultivars: *Aspergillus niger* (AN), *Fusarium moniliforme* (FM) and *Trichoderma longibrachiatum* (TL). The positive control was Ketoconazole 50 mg/ml. Results of the antifungal activity of chives, perilla, crown-daisy and guava leaves are shown in Table 4.

**Table 4:** Antifungal activity of leaves extract against fungi strains by agar well diffusion methods

	Inhibited zone (mm)		
	AN	FM	TL
<b>Ketoconazole (50 mg/ml)</b>	$18.43 \pm 1.2^{Aa}$	$30.36 \pm 0.4^{Ab}$	$17.43 \pm 0.8^{Aa}$
<b>Chives</b>	$24.22 \pm 0.9^{Ba}$	$12.52 \pm 1.7^{Bb}$	$9.82 \pm 1.2^{Bc}$
<b>Perilla</b>	-	-	-
<b>Crown-daisy</b>	-	-	-
<b>Guava</b>	-	-	-

The extract concentration was 50 mg/ml.



The diameter of the inhibited zone is expressed as mean  $\pm$  SD (mm).

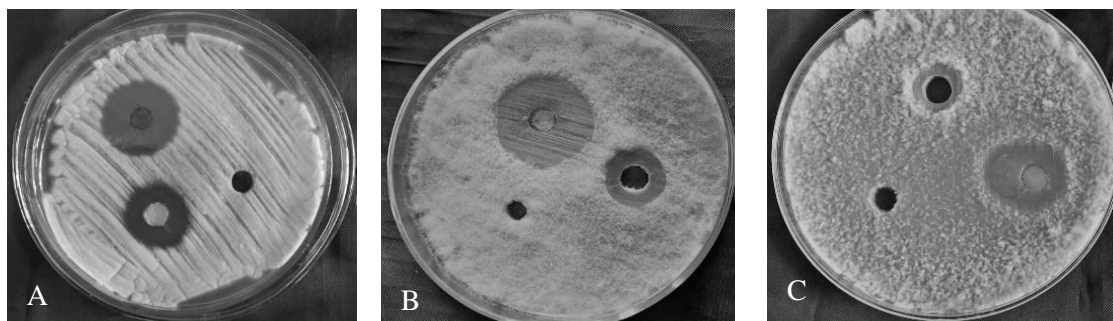
Symbol: " - " indicates extracts not resistant to microorganisms at concentrations above 50 mg/ml.

A-B: Different letters in the same column represent significant differences ( $P \leq 0.05$ ).

a-b: different letters in the same row represent significant differences ( $P \leq 0.05$ ).

The results from Table 4 show that only the extract from chive leaves had antifungal activity against cultivars. In particular, *Aspergillus niger* was the most inhibited strain with the inhibited zone about 24.22 mm ( $P \leq 0.05$ ). Followed by *Fusarium moniliforme* with the inhibited zone around 12.52 mm. The extract from chive showed the weakest activity against *Trichoderma longibrachiatum* with a diameter of 9.82 mm ( $P \leq 0.05$ ). Mei-chin Yin and Shih-ming Tsao (1999) extracted fresh chives with solvent acetic acid (at normal temperature) and water (at 65°C) and tested the antifungal activity with *Aspergillus niger*. The inhibited zones were 30.2 mm and 26.6 mm respectively.

The antifungal activity of the chives can be explained by the fact that the *Allium* family contained two major compounds, alicin and ajoene, which had antimicrobial activity (Yoshida *et al.*, 1987). Accordingly, the ajoene compound inhibits / kills cells by blocking G2 phase in the cell cycle. According to Yoshida *et al.*, 1999, the alicin compound in the *in vitro* test has effective against *Candida*, *Trichophyton*, *Microsporum*, *Cryptococcus* and *Epidermophyton*



**Figure 2:** Antifungal experiment of the extract from chive leaf

A. Experiment with *Aspergillus niger*

B. Experiment with *Fusarium moniliforme*

C. Experiment with *Trichoderma longibrachiatum*

### 3.4 Application of guava leaf extract in chicken wings samples

The experiment was tested with encoded samples as follows:

- C: Control sample
- BHT: Control sample treated with BHT 0.02% (v/w) (for antioxidant experiments)
- KS: Control sample treated with Potassium sorbate 0.5% (v/w) (for antimicrobial experiments)
- PG 0.5: Samples added with 0.5% guava leaf extract (v/w)
- PG 1: Samples added with 1% guava leaf extract (v/w)

### 3.4.1 pH

Figure 3 shows the effect of guava leaf extracts on pH value during storage of chicken wings. Samples were stored at 2 - 4°C for 10 days. The pH value is determined by AOAC (1995). Weigh 10g samples of anabolic meat in 100ml distilled water, assay after assimilation, and determine the pH of the filtrate by pH meter.

Result shows that the pH value of all samples decreased during storage. The most pH reduction during storage was observed in the PG 1 sample. Following was the sample of PG 0.5, BHT and finally was the control sample. Specifically, the pH value at 4 samples from day 0 to day 4 showed no statistically significant difference ( $P \leq 0.05$ ), between 6.0 and 6.22. From day 6 to day 10, the pH value of PG 0.5 and PG 1 samples were statistically significant difference ( $P \leq 0.05$ ), between 5.34 and 5.45, in comparison to that of 2 samples. Consequently, it can be concluded that the addition of extracts to the product affects the pH value of chicken wings during storage. Jayawardana *et al.*, (2011) also reported similarly on the reduction of the pH value of marinated sausages with moringa leaf extracts. In addition, a recent report showed a decrease in the pH of beef during cold storage (Ahn, J. *et al.*, 2002). In addition, Yunchuan, Junxiu and Yongkui (2014) also reported that there was a decrease in pH during the storage of Turkish fermented sausages by polyphenol extracted from tea. The decrease of pH may be due to the activity of the carbohydrate-causing bacteria that lead to the formation of organic acids, primarily lactic acid. However, the low pH value due to the formation of lactic acid is sometimes a positive factor in the production of sausages and processed meat products as it may contribute to an increase in shelf life due to inhibition of release bacterial growth at low pH.

### 3.4.2 Color values

Figure 4, 5 and 6 shows the effect of guava leaf extracts on color values ( $L^*$ ,  $a^*$  and  $b^*$ ) during storage of chicken wings. Samples were stored at 2 - 4°C for 10 days.

By the time of preservation, the  $L^*$  value of the samples tended to decrease, which means the product had darker color. The  $L^*$  values of the control samples, PG 0.5 and PG 1 were significantly ( $P \leq 0.05$ ) lower than that of the BHT sample. In particular, PG 1 sample has the lowest  $L^*$  value, followed by PG 0.5 and control sample. This indicates that the addition of the extract to the product has an effect on the  $L^*$  value during storage. The addition of the extract to the product makes the product darker than the control sample. The higher the extract volume, the darker the product. Only sample which had BHT supplementation was remained product color. This could be explained that the leaf extracts containing high phenolic content, they would be transformed into brown melanin products, thus reducing the brightness of the product (Mathew *et al.*, 1971).

The  $a^*$  value represents the color axis from green to red. Figure 5 shows the red color of the product decreasing during storage. Samples with were treated by leaf extracts (PG 0.5 and PG 1) and BHT had  $a^*$  value lower statistically significant ( $P \leq 0.05$ ) than that of control samples



during storage. This means that the color of PG 0.5, PG 1 and BHT samples were less red in comparison to that of control samples. Therefore, the addition of leaf extract to the product has an effect on the  $a^*$  value during storage. This result was consistent with the findings of Zhang *et al.*, 2016. Red meat color reduction during storage can be explained by the oxidation of meat pigments. Oxidation of free pigments causes iron oxidation reactions, altering myoglobin, which affects the color of meat products.

The  $b^*$  values of samples during storage were increased, the control sample had  $b^*$  value lower ( $P \leq 0.05$ ) than that of the other samples. This can be explained by the bright color compounds present in the leaf extracts (Zhang *et al.*, 2016). Therefore, the addition of the extract to the product has an effect on the  $b^*$  value during storage. Maqsood *et al.* (2012) also reported similar results in the study in addition to the extract of kiam wood to discolor fish sausages. Although color values are related to fat oxidation. However, the observation of these changes in this study is not indicative of protein oxidation or fat oxidation because dark yellow is also produced by the color of the extract.

#### 3.4.5 TBARS value

The TBARS value represents the amount of malondialdehyde - a product produced by secondary oxidation. The higher the TBARS value, the more malondialdehyde was produced and the faster the secondary lipid oxidation takes place, the lower the quality of the product. Figure 7 shows the effect of guava leaf extracts on TBARS values during storage of chicken wings. Samples were stored at 2 - 4°C for 10 days. Result shows that TBARS values in all samples increased during storage. The TBARS values in the treated samples (BHT, PG 0.5 and PG 1) were significantly ( $P < 0.05$ ) lower than that of control samples (C). The lowest TBARS value ( $P \leq 0.05$ ) was the PG 1 sample, followed by the PG 0.5 sample and the BHT sample. The results showed that the addition of extracts to the product had a positive effect in limiting oxidation during storage.

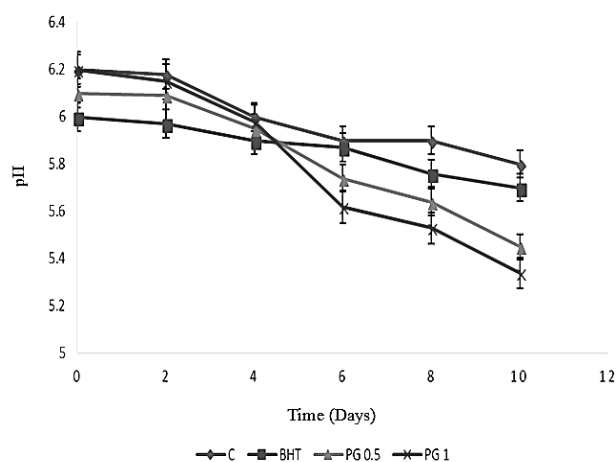
This result can be explained by natural antioxidants, which are believed to disrupt free radicals by transferring hydrogen from phenolic groups, resulting in the formation of a stable end product. . The results of this study are consistent with previous results from M.A.Lee *et al.*, 2010. The team found that mustard leaves have a high antioxidant activity due to the high content of phenolic compounds. Similarly, the extract of cloves and grape seed also has the effect of limiting oxidation, maintaining the TBARS value in the silver carp fillet during storage (Wenjiao Fan *et al.*, (2009)). In addition, H. Tajik *et al.*, (2014) have also shown the effect of antioxidant activity of grape seed extract during chicken preservation.

#### 3.4.6 Microbial analysis

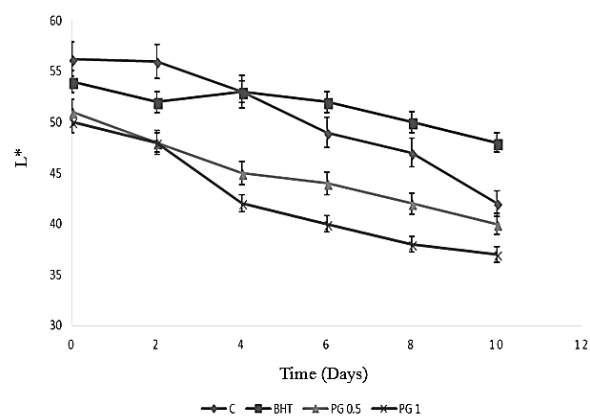
Figure 8 shows that during the 10 day storage period, TVC value of all samples had increased. In particular, TVC value of C and PG 0.5 samples were significant higher ( $P < 0.05$ ) than that of PG 1 and KS samples.

The initial LAB value was found to be 4.26 logCFU/g for all chicken wings samples. The LAB value increased in sample C and reached to 6.28 logCFU/g at the end of storage time (Figure 9). LAB value of KS, PG 0.5 and PG 1 samples were found to be lower ( $P < 0.05$ ) compared to that of sample C. In general, LAB of all 4 samples increased during storage. On day 10 of storage time, the LAB values were 6.18, 5.15, 5.52 and 5.34 logCFU/g for samples C, KS, PG 0.5 and PG 1 respectively. This is also reasonable with the results of pH value. During storage, the pH value decreased and the LAB value increased. Increasing the LAB value means lower pH of the product during storage. This can be explained by the activity of the bacteria which used carbohydrate lead to the formation of organic acids, primarily lactic acid. Lactic acid bacteria, which can grow both in the presence or absence of oxygen and are a significant part of the natural micro-organism of chicken. Lactic acid bacteria produced lactic acid, which caused a more sour taste than created the rotten flavor through the metabolism. The results of this study are consistent with those of H. Zhang et al. (2016). H.Zhang's team used clove and rosemary extracts mixed with chicken meat and monitored the LAB value after 12 days of storage (chill condition). The results showed that samples with extracts had LAB value increased significantly ( $p < 0.05$ ).

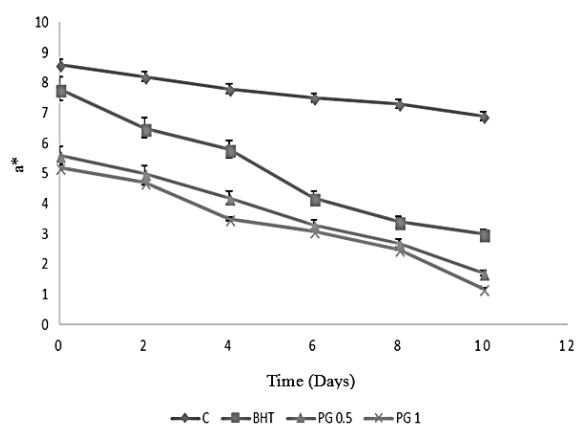
The amount of *S. aureus* was initially found to be 0.9 logCFU/g for all chicken wings samples. During the 10-day storage period, *S. aureus* generally increased with storage time (Figure 10). In particular, KS samples had a lower *S. aureus* value than that of ( $P \leq 0.05$ ) remaining samples. Samples C, PG 0.5 and PG 1 were generally not significantly different ( $P \leq 0.05$ ). On the 10<sup>th</sup> day of storage, the amount of *S. aureus* reached to 2.63, 1.65, 3.23 and 2.45 logCFU/g for samples C, KS, PG 0.5 and PG 1 respectively. Thus, the addition of extracts to the product contributed to limiting the increase of *S. aureus* during storage but ineffective by the addition of potassium sorbate. This can be explained by the fact that cold storage temperatures are not the optimal temperature for *S. aureus* as well as increased lactic acid production during storage also contributes to limiting the growth of *S. aureus*. The findings have some similarities with the study by Wenquian Yuan *et al.*, (2018). The team used the extract of *Syzygium antisepticum* (the same family with *Psidium guajava*) supplemented with processed chicken preserved at various temperature ranges. Results showed that, at chill temperature from 2 - 5 degrees C, *S.aureus* hardly increased during 5 days of survey.



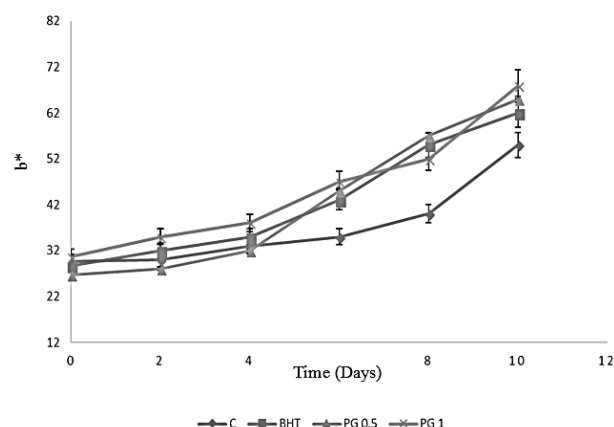
**Fig. 3:** Effect of guava leaf extracts on pH value during storage of chicken wings



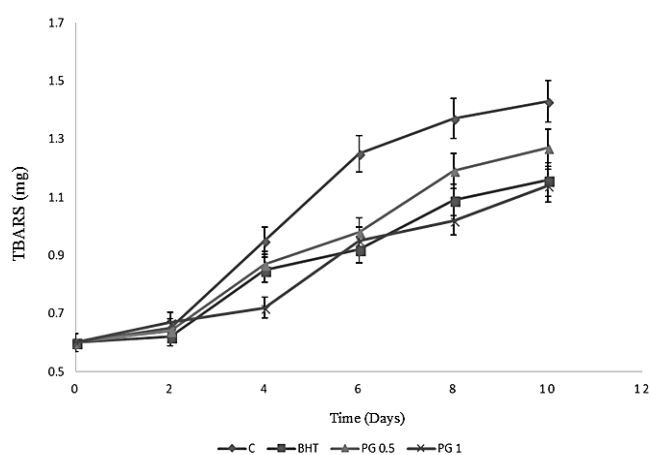
**Fig. 4:** Effect of guava leaf extracts on L\* value during storage of chicken wings



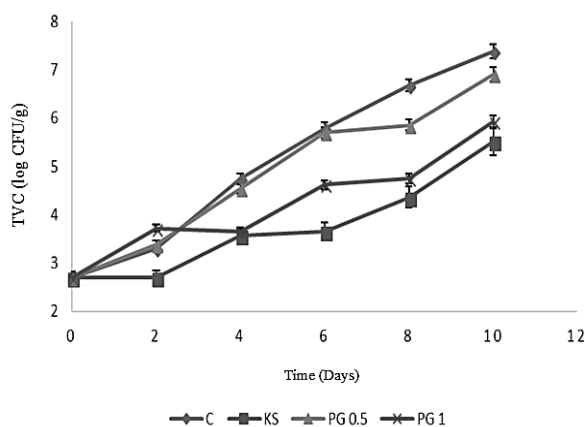
**Fig. 5:** Effect of guava leaf extracts on a\* value during storage of chicken wings



**Fig. 6:** Effect of guava leaf extracts on b\* value during storage of chicken wings



**Fig. 7:** Effect of guava leaf extracts on TBARS value during storage of chicken wings



**Fig. 8:** Effect of guava leaf extracts on TVC value during storage of chicken wings

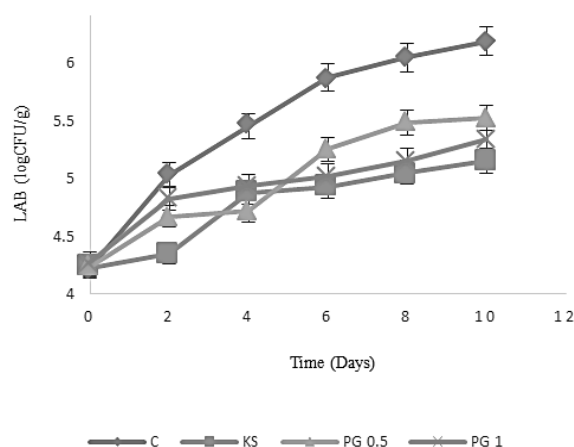


Fig. 9: Effect of guava leaf extracts on LAB value during storage of chicken wings

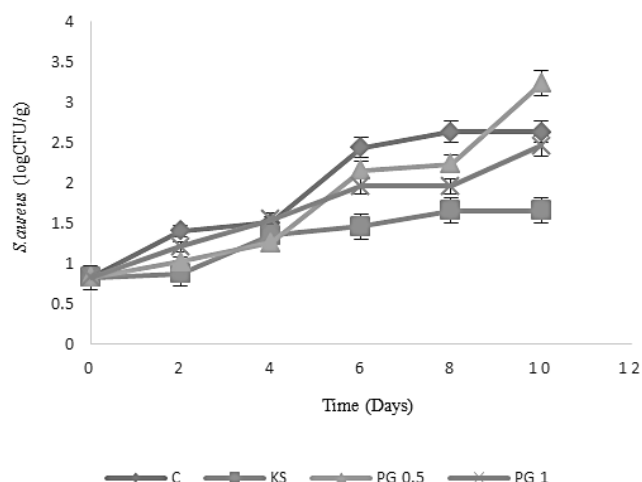


Fig. 10: Effect of guava leaf extracts on *S.aureus* value during storage of chicken wings

## 4 CONCLUSIONS

The present studies show that of all leaf types tested, guava leaves gave the best results in terms of antioxidant activity. Research shows the positive and potential benefits of using the extract from guava leaves as preservatives derived from nature. The extracts from the shallot have good antifungal results, especially with *Aspergillus niger*. (The diameter of the inhibited zone was greater than that of the positive control, Ketoconazole).

Study on the using of guava leaf extract has contributed to slow the oxidation of spiced chicken wings for 10 days preserved at temperature 2 - 4°C. Results showed that supplementation of extracts to reduce the TBARS values of poultry products during storage, also limiting the growth of *S. aureus* during storage. However, the addition of extracts to the product causes the pH decreasing of the product to and increasing the lactic acid bacteria value. Moreover, the addition of extracts to the product results in good storage but the product is darker, not looking good and organoleptic characteristics decreased.

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## THE DEVELOPMENT OF *CENTELLA ASIATICA* EXTRACT-LOADED PLGA NANOPARTICLES TO IMPROVE BIOAVAILABILITY

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### ABSTRACT

*Centella asiatica* (CA, Pennywort, Gotu kola) is used as a traditional drug widely in Asia. Since, CA crude extracts are poor absorption and decrease its bioavailability for over a period. The PLGA-nanoparticle (Poly lactic-co-glycolic acid) is used to overcome the problems. Therefore, the research was aimed to study the feasibility of CA crude ethanolic extract-loaded PLGA nanoparticles (NPs) production to improve bioavailability and study its biological properties.

The CA-PLGA-NPs prepared by double emulsion methods, Ratio (CA-Crude extract: PLGA): 1:2, 1:3, and 1:4 (w/w). The antioxidant activity of CA-PLGA-NPs was evaluated by using Total phenolic content (TPC). The modified agar well diffusion method was used to detect the antibacterial activity of CA-PLGA-NPs (100, 200, and 300 µg/ml) against 7 foodborne pathogens. The highest amount of antioxidant was CA-PLGA-NPs.1:4 ( $36.22 \pm 7.63^a$  µgGAE/mg.) For antibacterial activity, the CA-PLGS-NPs showed 2 – 3 times significantly higher than crude extract. CA-PLGA-NPs 1:3 (300 ug/mL.) shown the highest activity and it was significantly affected on gram-positive bacteria *S. aureus* and *B. cereus* ( $1.02 \pm 0.3^b$  and  $0.78 \pm 0.06^b$  cm.) ( $p < 0.05$ ). The entrapment efficiency of CA-PLGA-NPs. rank from high to low were 1:4 ,1:3 and 1:2 respectively ( $92.61 \pm 5.09$  %,  $63.89 \pm 4.13$  %, and  $30.61 \pm 2.41$  %) ( $p < 0.05$ ). Solubility parts, the highest and lowest solubility in the water are CA-crude extracts ( $682.89 \pm 22.28$  µg/mL) and CA-PLGA-NPs. 1:2 ( $480.97 \pm 37.7$  µg/mL). CA-PLGA-NPs. also showed stability and releasing of CA in PBS (0.01M, pH 2.0 and pH 7.4) for 6 hours. Moreover, at pH 7.4 the sample tend to release CA faster, more stable and less time consuming than at pH 2.0. These results indicated that CA-PLGA-NPs provide the promising to increase the bioavailability of CA, which can be developed into the broad spectrum of usage.

**Keywords:** *Centella asiatica*, PLGA, Nanoparticle, Antibacterial, Antioxidant, Entrapment and Loading, Release Kinetic



## 2 INTRODUCTION

*Centella asiatica* (CA) is a tropical medicinal plant from Apiaceae family that mostly found in South East Asia and spread through Western part of the world. CA is used as traditional drug to reduce the blood pressure, cure the fresh wound, heal bruised and diuretic [1]. Moreover, it has been reported for its bioavailability activities, antimicrobial activity, antioxidant activity, anti-inflammatory activity, wound healing activity and anti-cancer activity [2]. The major compound active of *C. asiatica* are polyphenols and triterpenes [3]. Furthermore, CA could inhibit the growth of *Bacillus cereus* and *Listeria monocytogenes* at normal, osmotic stress, and high acidic conditions, the growth of pathogenic bacteria in intestines and the growth of both Gram-positive and Gram-negative bacteria [4]-[7]. As the benefits that mentioned above, herbal extract market in Thailand and the Western country has been continuously growing due to increasing of the health concern and the aged society. Therefore, the *C. asiatica* extract had high potential to be used as raw material in pharmaceutical, cosmetic, personal care, and food industry.

Despite CA carry many beneficial activities but it is difficult for water-soluble biological active compounds to enter through cell membrane of both human and pathogenic microorganism, which has hydrophobic characteristic [8]. These affect the biological absorption activity and loading system of both in human and in microbial.

One of the best ways to improve drug delivery system of crude extract, increase the efficiency of the drug is the nanotechnology. This technology has been used to improve drug bioavailability and drug delivery system [10-12]. Poly (lactic-co-glycolic acid) (PLGA) is one of the most effective biodegradable polymeric nanoparticles (NPs). It has been approved by the US FDA to use in drug delivery systems due to controlled and sustained- release properties, low toxicity, and biocompatibility with tissue and cells [13]. It is most popular among the various available biodegradable polymers because of its long clinical experience, favorable degradation characteristics and possibilities for sustained drug delivery [14]

The research aimed to study the feasibility of *C. asiatica* extract-loaded PLGA nanoparticles (CA-PLGA-NPs) production to improve it bioavailability.

## 3 MATERIALS AND METHODS

### 3.1 Preparation of *C. asiatica* (CA)

Fresh CA was purchased from Bangkapi markets in Bangkok, Thailand. The aerial part of CA was used. Fresh CA were washed with tap water and cut into small pieces. Then it was air dry in oven (Memmert UM500) at 45°C. The dried samples were finely ground into powder. The powder was kept at 4°C before used [8].

### 3.2 Preparation of CA Crude Ethanol Extract

Ca powder is extracted with ethanol using 1:10 ratio (g/mL). The mixtures are macerated at room temperature, 120 rpm, for 48 hours and then are filtered using Whatman filter paper no.4. The CA extracts were evaporated at 45°C by rotary evaporators (BUCHSI Rotavapor R-205). The crude was stored at -20°C prior to use in preparation of CA-PLGA-NPs [8].

### 3.3 Preparation of CA Poly (DL-lactide-co-glycolide) (PLGA) Nanoparticle (NPs)

CA-PLGA-NPs is prepared according to water-in-oil-in-water (w/o/w) solvent evaporation techniques [10]. The 200 mg of PLGA is dissolved in 20 ml acetone to obtain a uniform PLGA solution. The addition of ca crude extract 1:2, 1:3 and 1:4 ration is added to PLGA solution and stirred at 25 °c for 30 min. The mixture is emulsified with 40 ml of PVA solution (1% w/v) by rotating at 300 rpm. And homogenize at 19,000 rpm for 5 min. To generate the final s/o/w emulsion. The solvent was evaporated out at 50 °c for 30 min. Larger aggregates and free PLGA/pva polymers are removed by centrifugation. Finally, the mixture is lyophilized and stored at 4 °c.

### 3.4 Antimicrobial Activity

The modified agar well diffusion method [8] is used. The 100 µL of bacteria (approximately  $1.5 \times 10^8$  CFU/mL) was spread on Mueller-Hinton Agar (MHA) plate. The 50 µL of CA crude ethanolic extract and CA-NPs at concentration 100, 200, and 300 µg/mL diluted with distilled water that were used to test antibacterial activity against *Escherichia coli* ATCC25822, *Streptococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone. The 20 µL of 50 mg/mL penicillin G was used as positive control. The inhibition zones were measured to determine the effectiveness of the CA crude extract and CA-NPs against each bacterium.

### 3.5 Antioxidant Activity - Total Phenolic Content (TPC)

The Modified Folin–Ciocalteu Method [15] was used for total phenolic content determination of CA crude ethanolic extract and CA-NPs. The 20 µL of 10 mg/mL CA crude ethanolic extract and CA-NPs was added to 1.58 mL distilled water and 100 µL Folin–Ciocalteu phenol reagent. The mixture was then stand for 8 minutes 30 seconds and 300 µL saturated sodium carbonate solution was added to the mixture. After that, the mixture was incubated without light at room temperature for 30 minutes and observed optical density (OD) at 765 nm. The results were expressed as microgram gallic acid equivalent (µgGAE/mL). The experiment was done in triplicate and three replications independently.

### **3.6 Entrapment and Loading Efficiency**

CA crude ethanolic extract was run absorbance spectrum to find the best the wavelength ( $\lambda_{\text{max}}$ ) at which the absorbance is the greatest by UV-vis spectrophotometer. The 2 mg CA-NPs were dissolved in 1 mL methanol and gently shaken for 24 hours at 37 °c to completely extract CA crude ethanolic extract to methanol then the solutions were centrifuged at 12,000 rpm for 10 min, and the supernatant was kept and measured optical density (OD) by a UV-vis spectrophotometer at  $\lambda_{\text{max}}$ . The amount of CA crude ethanolic extract entrapped and loaded in CA-NPs is express as entrapment efficiency and loading efficiency calculated as follows [16]:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of CA crude extract in nanoparticles}}{\text{Amount of total feeding CA crude extract}} \times 100 \quad (1)$$

$$\text{Loading efficiency (\%)} = \frac{\text{Amount of CA crude extract in nanoparticles}}{\text{Amount of nanoparticles}} \times 100 \quad (2)$$

All measurements were done in triplicate and three replications independently.

### **3.7 Release Kinetic in Vitro**

Release kinetic [16] methodology was modified. The release of CA-crude ethanolic extract from CA-PLGA-NPs was done by dissolving 20 mg of CA-PLGA-NPs in 15 mL artificial gastric juice (0.01 m pbs pH 2.0) and intestinal juice without enzymes (0.01 m pbs pH 7.4). The mixture is incubated at 37 °c at 200 rpm. At designated time points (0, 0.5, 1, 2, 3, 4, 5, 6 hours), mixture is sampled and centrifuged at 3000 rpm for 10 min. The pellet is resuspended in 100  $\mu\text{L}$  of methanol to determine the amount of CA-crude ethanolic extract released by measuring optical density (OD) by UV-vis spectrophotometer at  $\lambda_{\text{max}}$ . All measurements were done in triplicate and three replications independently.

### **3.8 Statistical Analysis and Experimental Design**

All experiments were conducted in triplicate and three replications independently. The statistical analysis was accomplished by using ANOVA with Duncan's multiple range tests ( $p < 0.05$ ) by SAS software version 9.4.

## 4 RESULTS AND DISCUSSION

### 4.1 Antimicrobial Activity

**Table 1:** Antibacterial activity as inhibition zone (cm.) of CA-PLGA-NPs and crude extract against 7 different microorganisms

Sample	Conc. (µg/mL)	Inhibition zone (cm.)						
		<i>E. coli</i>	<i>S. aureus</i>	<i>B. cereus</i>	<i>B. subtilis</i>	<i>ST</i>	<i>SE</i>	<i>SUS</i>
CA-PLGA-NPs 1:2	100	0.6 ± 0.15 <sup>bc,BC</sup>	0.97 ± 0.14 <sup>b,B</sup>	0.44 ± 0.09 <sup>c,C</sup>	0.5 ± 0.00 <sup>b,B</sup>	0.59 ± 0.23 <sup>bc,BC</sup>	0.36 ± 0.20 <sup>c,C</sup>	0.46 ± 0.34 <sup>b,B</sup>
	200	0.75 ± 0.35 <sup>b,B</sup>	0.95 ± 0.18 <sup>b,B</sup>	0.47 ± 0.05 <sup>c,C</sup>	0.42 ± 0.12 <sup>bc,BC</sup>	0.6 ± 0.24 <sup>bc,BC</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.58 ± 0.11 <sup>ab,AB</sup>
	300	0.41 ± 0.27 <sup>c,C</sup>	1 ± 0.18 <sup>b,B</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.51 ± 0.01 <sup>b,B</sup>	0.6 ± 0.28 <sup>bc,BC</sup>	0.58 ± 0.20 <sup>b,B</sup>	0.64 ± 0.26 <sup>a,A</sup>
CA-PLGA-NPs 1:3	100	0.76 ± 0.54 <sup>a,A</sup>	1.01 ± 0.18 <sup>b,B</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.47 ± 0.00 <sup>b,B</sup>	0.55 ± 0.35 <sup>c,C</sup>	0.4 ± 0.14 <sup>c,C</sup>	0.38 ± 0.22 <sup>c,C</sup>
	200	0.58 ± 0.25 <sup>b,B</sup>	0.99 ± 0.26 <sup>b,B</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.5 ± 0.00 <sup>b,B</sup>	0.62 ± 0.45 <sup>bc,BC</sup>	0.4 ± 0.14 <sup>c,C</sup>	0.5 ± 0.28 <sup>ab,AB</sup>
	300	0.78 ± 0.49 <sup>ab,AB</sup>	1.02 ± 0.3 <sup>b,B</sup>	0.78 ± 0.06 <sup>b,B</sup>	0.45 ± 0.07 <sup>bc,BC</sup>	0.76 ± 0.55 <sup>b,B</sup>	0.42 ± 0.02 <sup>c,C</sup>	0.68 ± 0.35 <sup>a,A</sup>
CA-PLGA-NPs 1:4	100	0.37 ± 0.09 <sup>d,D</sup>	0.74 ± 0.34 <sup>c,C</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.5 ± 0.00 <sup>b,B</sup>	0.73 ± 0.32 <sup>b,B</sup>	0.37 ± 0.00 <sup>c,C</sup>	0.54 ± 0.23 <sup>b,B</sup>
	200	0.3 ± 0.00 <sup>d,D</sup>	1 ± 0.1 <sup>b,B</sup>	0.5 ± 0.00 <sup>bc,BC</sup>	0.42 ± 0.12 <sup>bc,BC</sup>	0.68 ± 0.25 <sup>b,B</sup>	0.4 ± 0.14 <sup>c,C</sup>	0.64 ± 0.18 <sup>a,A</sup>
	300	0.37 ± 0.05 <sup>d,D</sup>	0.99 ± 0.2 <sup>b,B</sup>	0.42 ± 0.26 <sup>c,C</sup>	0.5 ± 0.00 <sup>b,B</sup>	0.69 ± 0.27 <sup>b,B</sup>	0.44 ± 0.05 <sup>c,C</sup>	0.64 ± 0.23 <sup>a,A</sup>
Crude	100	0.35 ± 0.07 <sup>c,C</sup>	0.37 ± 0.09 <sup>d,D</sup>	0.42 ± 0.02 <sup>c,C</sup>	0.42 ± 0.42 <sup>b,B</sup>	0.47 ± 0.59 <sup>a,A</sup>	0.31 ± 0.54 <sup>b,B</sup>	0.29 ± 0.41 <sup>c,C</sup>
	200	0.32 ± 0.37 <sup>ab,AB</sup>	0.44 ± 0.06 <sup>c,C</sup>	0.59 ± 0.27 <sup>bc,BC</sup>	0.45 ± 0.37 <sup>b,B</sup>	0.42 ± 0.40 <sup>b,B</sup>	0.28 ± 0.46 <sup>b,B</sup>	0.46 ± 0.23 <sup>b,B</sup>
	300	0.52 ± 0.33 <sup>a,A</sup>	0.62 ± 0.02 <sup>c,C</sup>	0.60 ± 0.07 <sup>b,B</sup>	0.29 ± 0.54 <sup>b,B</sup>	0.48 ± 0.76 <sup>a,A</sup>	0.19 ± 0.60 <sup>b,B</sup>	1.36 ± 0.06 <sup>c,C</sup>

Note: Different letter (A, B, C, a, b, c) of superscript within a row and column showed significant different at  $p < 0.05$ , respectively. Full name of the microorganism:

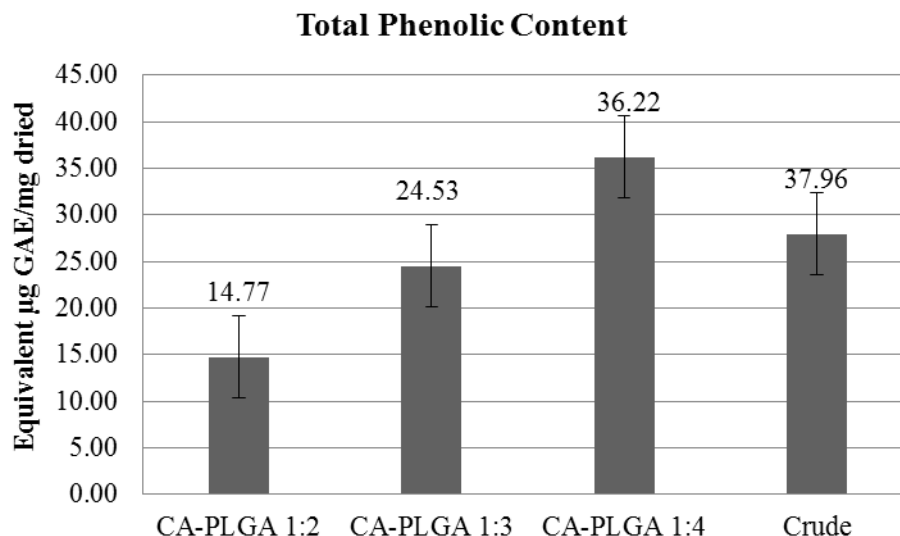
*Escherichia coli* ATCC25822, *Streptococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Salmonella enterica* Typhimurium U302 (DT104b) (ST), *S. enterica* Enteritidis (human)(SE), and *S. enterica* 4,5,12:i:- (human) US clone(SUS).

The modified agar well diffusion method was used for evaluating antibacterial activity of CA-PLGA-NPs and CA with different concentration (100, 200, and 300 µl/ml) against seven foodborne pathogens. The result from table 2. shown the antibacterial activities trend of CA-PLGS-NPs was about 2 – 3 times significantly higher than CA crude ethanolic extracts ( $p < 0.05$ ). Especially for *S. aureus* ( $1.02 \pm 0.3^b$  cm.) which shown distinctive result than other bacteria and this maximum inhibitory effect is similar with the study by R. Perumal Samy, Ignacimuthu and Vincent TK Chow that showed the CA crude extracts was very effective in inhibiting the growth of all the test microorganisms especially *S. aureus*, *B. cereus*, *E. coli*, *B. subtilis* and *Salmonella typhi* respectively.

The growth of the bacteria was inhibited by the triterpenes in CA. Due to it is the polar compounds which ionization of molecule combine with the absorption of polyphenols to bacterial membranes made its membranes became disrupted. These active compounds may be able to penetrate the thick cell walls through general diffusion channels formed by the bacterial porins present therein [17] and affect the bacterial enzymes that are responsible for survival of microbe [18] resulting in cellular lysis. However, these extracts do not affect some of the bacteria which may be due to hindrance of penetration through the outer cell wall and absence of specific enzyme in the bacteria. This mode of action of the plant extract against the specific bacteria may be due to its secondary mode of action against the bacterial enzymes instead of acting on the cell wall of the bacteria.

When comparing between gram-positive and gram-negative bacteria, the CA-PLGA-NPs tend to have more significantly effect on gram-positive bacteria which are *S. aureus* and *B. cereus* ( $1.02 \pm 0.3^b$  and  $0.78 \pm 0.06^b$  cm.). By the reason of gram-positive bacteria was more sensitive than gram-negatives [19] lead to easier invaded by the active compounds of the CA. Furthermore, comparing the structure of the cell membrane among gram-positive and gram-negative bacteria. The gram-positive bacteria, there is an inner membrane which is surrounded by a thick cell wall made of peptidoglycan [20] which found mostly hydrophilic characteristic and due to the CA-crude extracts also contain hydrophilic active compound therefore, this make the water-soluble biological active compounds easily to penetrate through the thick peptidoglycan of the gram-positive bacteria and inhibit it. In the other hand, the gram-negative bacteria are tending to have more protective layers which including an inner membrane are followed by a thin peptidoglycan layer and an outer membrane. Additionally, inhibition of microbes by well agar diffusion method is also influenced by concentration of extract, duration of exposure and the microbes tested [21].

## 4.2 Antioxidant Activity - Total Phenolic Content (TPC)



**Figure 1:** TPC of CA-PLGA-NPs. and Crude extract expressed as equivalent µg GAE ( $p < 0.05$ )

The antioxidant activity of all CA-PLGA-NPs and CA crude extract were statistically analyzed to see the difference between each sample that was prepared from different preparation methods and ratios.

As showed in Figure 1. TPC of CA-PLGA-NPs and crude extract were determined in comparison with standard gallic acid and the results were expressed in terms of µg GAE/mg dried weight. The highest antioxidant activity represented by the amount of phenolic content of CA-PLGA-NPs. was  $36.22 \pm 7.63^a$  µgGAE/mg using ratio 1:4. The amount of phenolic content of CA-PLGA-NPs. did not show significant different between each ratio 1:2, 1:3 and crude sample ( $P < 0.05$ ). In addition, according to Nazck and Shahidi (2004) [22], Follin-Ciocalteau reagent that use to test the TPC was not specific and can detects all phenolic groups found in the samples.

## 4.3 Entrapment and Loading Efficiency

**Table 2:** Percentage entrapment and loading efficiency of CA-PLGA-NPs.

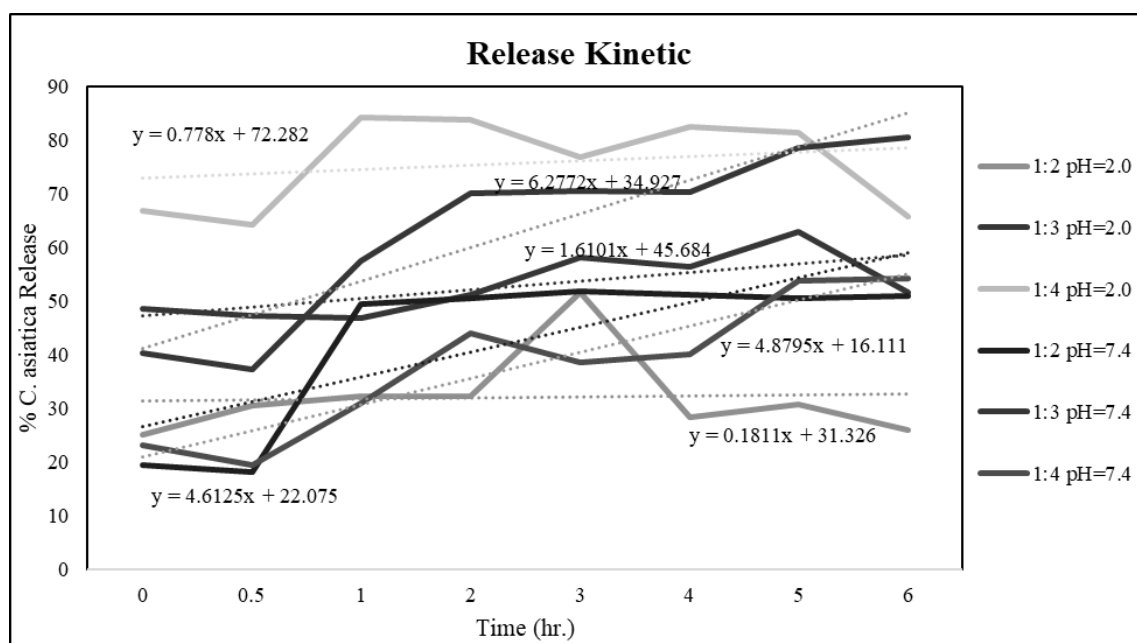
Sample	Entrapment efficiency (%)	Loading Efficiency (%)
CA-PLGA-NPs 1:2	$30.61 \pm 2.41^c$	$26.02 \pm 2.05^c$
CA-PLGA-NPs 1:3	$63.89 \pm 4.13^b$	$35.14 \pm 2.27^b$
CA-PLGA-NPs 1:4	$92.61 \pm 5.09^a$	$37.04 \pm 2.04^a$

*Note: Different superscript within a column showed significant different at  $p < 0.05$*

As showed in Table 2. CA-PLGA-NPs. at ratio 1:4 has entrapment efficiency significantly higher than ratio 1:3 and 1:2. There are significantly difference in both entrapment and loading efficiency among ratio of CA-PLGA-NPs. Moreover, CA-PLGA-NPs. at ratio 1:4 also has

highest percentage followed by 1:3 and 1:2 in both results. In addition, the reason why CA-PLGA-NPs. at ratio 1:2 has the lowest entrapment efficiency (%) might cause by the process of forming PLGA nanoparticle which is the double-emulsion (w/o/w) method. This method is the large size of the nanoparticles formed and leakage of the hydrophilic active component [23], responsible for low entrapment efficiencies. The coalescence (particles merge together) and Ostwald ripening (Change of an inhomogeneous structure over time, i.e., small crystals or sol particles dissolve, and redeposit onto larger crystals or sol particles [24] are the two important mechanisms that destabilize the double-emulsion droplet, and the diffusion through the organic phase of the hydrophilic active component is the main mechanism responsible of low levels of entrapped active component [25].

#### 4.4 Release Kinetic in Vitro



**Figure 2:** Release rate of CA from CA-PLGA-NPs in PBS (pH 2.0 and pH 7.4) at 37°C for 6 hrs.

In the study of release kinetic in vitro which is based on general transit time of drugs in the GI tract and the tested sample are CA-PLGA-NPs at ratio 1:2, 1:3, and 1:4. Moreover, artificial gastric juice (0.01 M PBS pH 2.0) and artificial intestinal juice (0.01 M PBS pH 7.4) were used to imitate the environment in the human stomach and intestine. The cumulative percentage of release CA was determined as showed in Figure 2. The release rate of CA from CA-PLGA-NPs at the same ratio in the gastric juice (pH 2.0) was tending to slower than intestinal juice (pH 7.4) except for the CA-PLGA-NPs. ratio 1:4. Furthermore, arrange from the fastest to slowest CA release for pH 2.0 are CA-PLGA-NPs. ratio 1:4, 1:3 and 1:2 respectively. And for pH 7.4 are CA-PLGA-NPs. ratio 1:3, 1:2 and 1:4 respectively. At CA-PLGA-NPs.1:3 at pH 7.4 % CA release is the highest (> 80%) at the final 6 hours. However, comparing between CA-PLGA-NPs.1:3 at pH 7.4 and CA-PLGA-NPs.1:4 at pH 2.0, the CA-PLGA-NPs.1:4 tend to have above 70 % CA



release throughout 0 to 6 hours. CA-PLGA-NPs. for all ratio and for both pH started to release CA at 0 to 0.5 hours. However, at pH 7.4 the sample tend to release CA faster and stable than at pH 2.0. CA-PLGA-NPs can be released in very low pH, therefore, it can be applied to the oral medicine.

## 5 CONCLUSIONS

The present study of CA-PLGA-NPs indicated impressive result on the antibacterial and antioxidant activity. The antibacterial activity of CA-crude ethanolic extracts can be improved by the encapsulation process of the CA-PLAG-NPs to against the human pathogens (2 – 3 times higher). Especially, on gram-positive bacteria *S. aureus* and *B. cereus*. Moreover, the total phenolic content of the CA-PLGA-NPs and the crude extract shown no significantly different among each other ( $p < 0.05$ ). CA-PLGA-NPs. at ratio 1:4 shown the best result on the entrapment and loading efficiency. Furthermore, the CA-PLGA-NPs releases in very low pH, therefore, it can be applied to the oral medicine. And at pH 7.4 the sample tend to release CA faster, more stable and less time consuming than at pH 2.0. These results provide the promising to increase the bioavailability of CA, which can be developed into the broad spectrum of usage. And since, PLGA-NPs can improve drug delivery system of the crude extract and increase the efficiency of the drug these can lead to reducing cost from purification steps and can be applied to use in high valued industry products. Lastly, the development of CA-PLGA-NPs would be a promising improving bioavailability of CA-crude extract.

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## **SYNTHESIS COMPLEXS OF CURCUMIN WITH FE (III), CU (II), ZN (II) AND INITIALLY APPLIED IN THE TREATMENT OF BURNS IN MICE**

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### **ABSTRACT**

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], was extracted from *curcuma longa*. Its metal complexes (M = Fe, Cu, Zn) were prepared and characterized by IR and UV-vis spectroscopy. The result showed that curcumin coordinates with metal ions in bidentate mode in deprotonated form. A 1:1 complex between of CUR and Fe(III) at pH = 7.5 with new peak at 510nm was formed. The complex of CUR with Cu (II) ion was also formed at a ratio of 1:1 at pH = 7.5 and had a maximum absorbance at 505 nm. Meanwhile, the Zn (II) complex with CUR was also found at pH = 5.6 and the maximum absorption wavelength is 428nm. IR spectral values also demonstrated the formation of complexes through the oscillator signal changes of functional groups. Complexes had also been tested in mouse burn aid support against pure CUR itself. The results showed that the wound healing time in mice was 22 days, when CUR and complexes were applied 3 times daily. Time for wound recovery in mice skin was 18 days for CUR, 15 days for copper complex, 15 days for zinc complex. Specially, CUR-Fe complex required only 12 days to completely recover burn on mice skin.

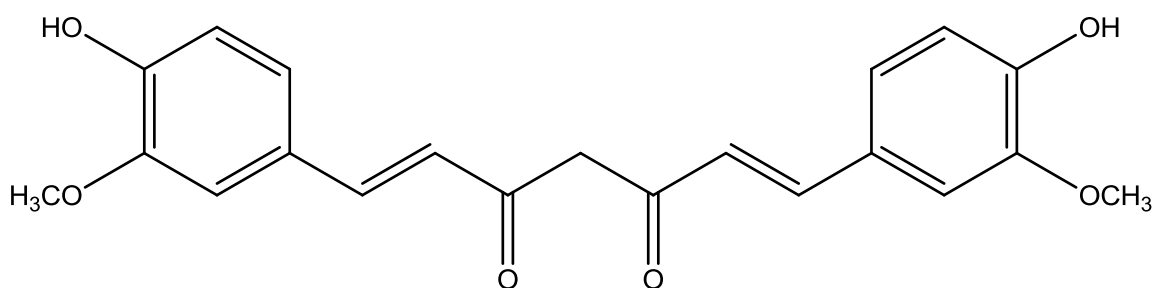
### **1 INTRODUCTION**

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadien-3,5-dione, is a lipophilic molecule that rapidly permeates cell membrane (E.Jaruga et al). Typical extract of *Curcuma longa L.* contains the structures I to III: (I) diferuloylmethane/curcumin (curcumin I, 75%), (II) demethoxycurcumin (curcuminII, 20%), and (III) bisdemethoxycurcumin (curcumin III, 5%) (S. Sreejayan and M.N. A. Rao) (Figure1). Curcumin is an active ingredient in the herbal remedy and dietary spice turmeric [7]. Many researchers have worked on curcumin due to its various therapeutic effects on different diseases. Shortly, curcumin has received attention mostly due to its antioxidant, anti-inflammatory, antitumoral, apoptosis-inducing, and antiangiogenesis effects, which were reported in many investigations. It acts on multiple targets in cellular pathways making this agent able to perform multiple actions [2].

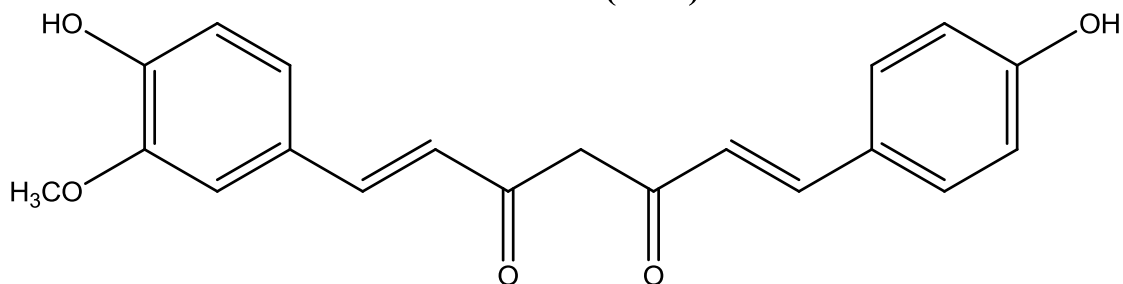
The simple molecular structure along with the relative density of functional groups in curcumin provides researchers with an outstanding target for structure-activity relationship and lead

optimization studies. The structural analogues of curcumin have been reported to enhance the rate of absorption with a peak plasma half-life [1].

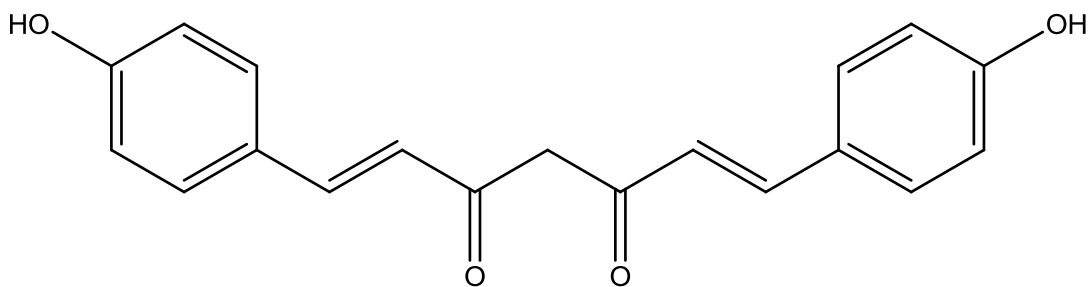
Recent investigations have considered curcumin a lead compound or designing new chemotherapeutic agents for treatment of cancers including colon cancers [9], prostate cancers [5] and other conditions with indication of chemotherapy [15]. Curcumin is remarkably well tolerated, but its bioavailability is poor. It does not appear to be toxic to animals [18] or humans [8], even at high doses. Recent studies have discussed poor bioavailability of curcumin because of poor absorption, rapid metabolism, and rapid systemic elimination [14]; however, comprehensive pharmacokinetic data are still missing. In a study done by Yang et al [16], they reported 1% bioavailability for oral administration of curcumin in rats. On the elimination of curcumin, an investigation in rat model demonstrated that after oral administration of 1g/kg of curcumin, more than 75% was excreted in feces and negligible amount of curcumin was detected in urine. Additionally, FDA has declared curcumin as "generally safe." Although curcumin showed a wide variety of useful pharmacological effects and has been found to be quite safe in both animals and humans, there are some studies concerning its toxicity [10]. In spite of these advantages, curcumin has poor water solubility; as a consequence, it reveals solubility limited bioavailability, which makes it a class II drug in the biopharmaceutics classification system [13]. Additionally, due to its rapid intestinal and hepatic metabolism, about 60% to 70% of an oral dose of curcumin gets eliminated by the feces [11]. As mentioned above, curcumin has been proven to be effective in treatment of different diseases with low toxicity to human and animals. It is extremely safe upon oral administration even at very high doses; however, it is limited due to its poor bioavailability, stability, low solubility, and rapid degradation and metabolism. Overcoming these problems has been the main goal of many studies over the past three decades. Since curcumin was demonstrated to have poor bioavailability and selectivity [4], numerous analogues of this material have been introduced and tested in order to evaluate their activities against known biological targets and to also improve their bioavailability, selectivity, and stability [6]. In addition, several approaches were introduced to improve the bioavailability, to increase the plasma concentration, and to enhance the cellular permeability and resistance to metabolic processes of curcumin. There are many reports about curcumin and its metal ion complexes and their structures and free radical scavenging ability [19]. However, the components, structures of the metal-curcumin complexes are still necessary to be investigated clearly for understanding the properties of the complexes. In present work, these issues of curcumin and its complexes with  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Fe}^{3+}$  ions were investigated, and these complexes also were used to treat for burn of rats.



(1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione  
**Curcumin (CUR)**



(1*E*,6*E*)-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione  
**Demethoxycurcumin (DCM)**



(1*E*,6*E*)-1,7-bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione  
**Bisdemethoxycurcumin (BDMC)**

**Figure 1:** Structure of 3 curcuminoids

## 2 MATERIALS AND METHODS

### 2.1 Material

Curcumin (90%), iron (III) chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\geq 99\%$ ), Copper (II) chloride ( $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\geq 99\%$ ), Zinc (II) chloride ( $\text{ZnCl}_2$ ,  $\geq 98\%$ ), Ethanol ( $\text{C}_2\text{H}_5\text{OH}$ ,  $\geq 99,5\%$ ), acid acetic ( $\text{CH}_3\text{COOH}$ ,  $\geq 99,5\%$ ), Sodium acetate ( $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ ,  $\geq 99\%$ ).

### 2.2 Method

*2.1.1 The complex of CUR were synthesized by Zao method [19].*

Curcumin powder and metal ions were completely dissolved with ethanol. Solution containing metal ions were slowly added to the curcumin solution and the mixture was stirred during 2 hours. pH was adjusted by adding ammonia solution. The mixture was centrifuged at 5000 rpm.

After 10 minutes, the complex was collected, then the complex was dried at 50 °C for 2 hours. The curcumin-metal complex was obtained in crystalline form. After filtration and purification, the obtained products were yellow-brown to black.

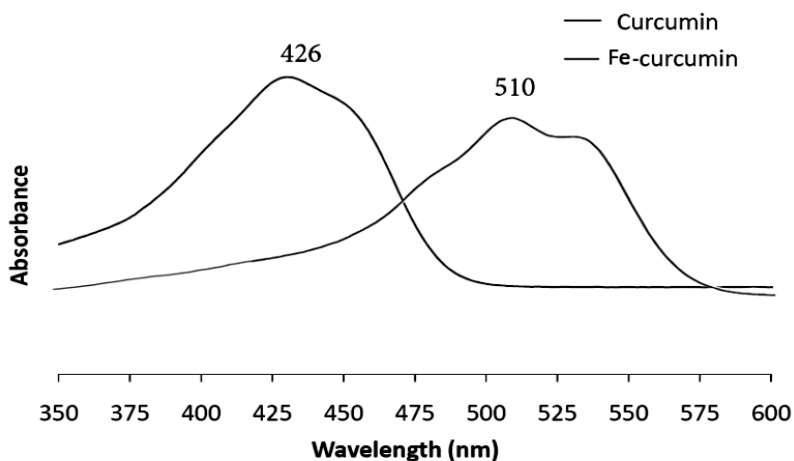
*2.1.2. UV absorption spectra of curcumin and its complexes in DMSO were recorded on Perkin Elemer Lambda 35 UV/Vis spectrophotometer. The resolution of the spectrum was 1 nm and the observed absorption wavelength was from 350 to 500 nm.*

*2.1.3. IR spectroscopy IR spectra of curcumin and its complexes were recorded on Nicolet Magna 550 spectrometer in KBr discs with resolution of 4 cm<sup>-1</sup> and scans of 32. The spectral range was from 4000 to 400 cm<sup>-1</sup>.*

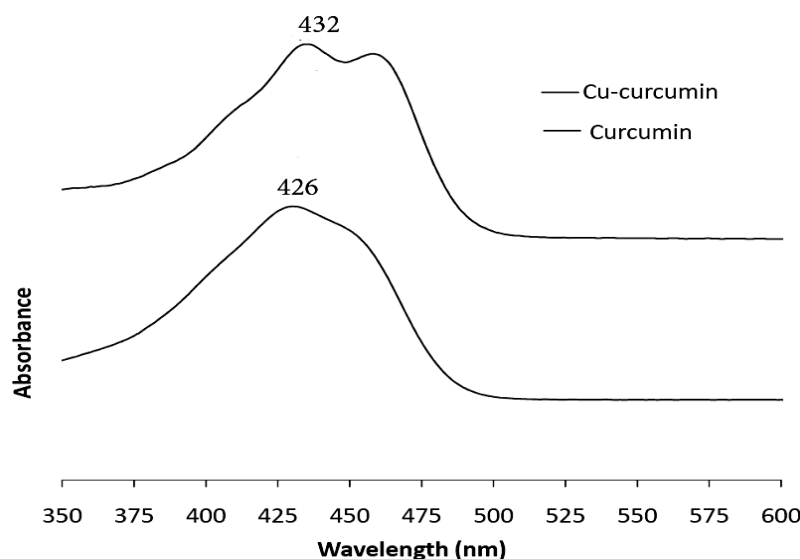
### 3 RESULTS AND DISCUSSION

#### 3.1 Absorption spectra of Curcumin complexes

The absorption spectra of the reagent CUR and its complexes under the optimum conditions were shown in Figure.2 and Figure 3. In the figure, the spectra of CUR and these complexes were showed respectively against water blank. As the observation, the maximum absorption peak of the CUR lies at 424 nm, corresponding  $\pi$  to  $\pi^*$  transition of the -C= O- bond, which was in accordance with typical spectra as observed by other worker [19]. Whereas, the absorption peak of the complexes was located at 432 nm (CUR-Cu), 510 nm (CUR-Fe) and 428 nm (CUR-Zn). Hence, a very large wavelength change ( $\lambda= 86$  nm) was obtained.



**Figure 2:** Absorption of spectra of CUR (a) and CUR-Fe(III) (b)



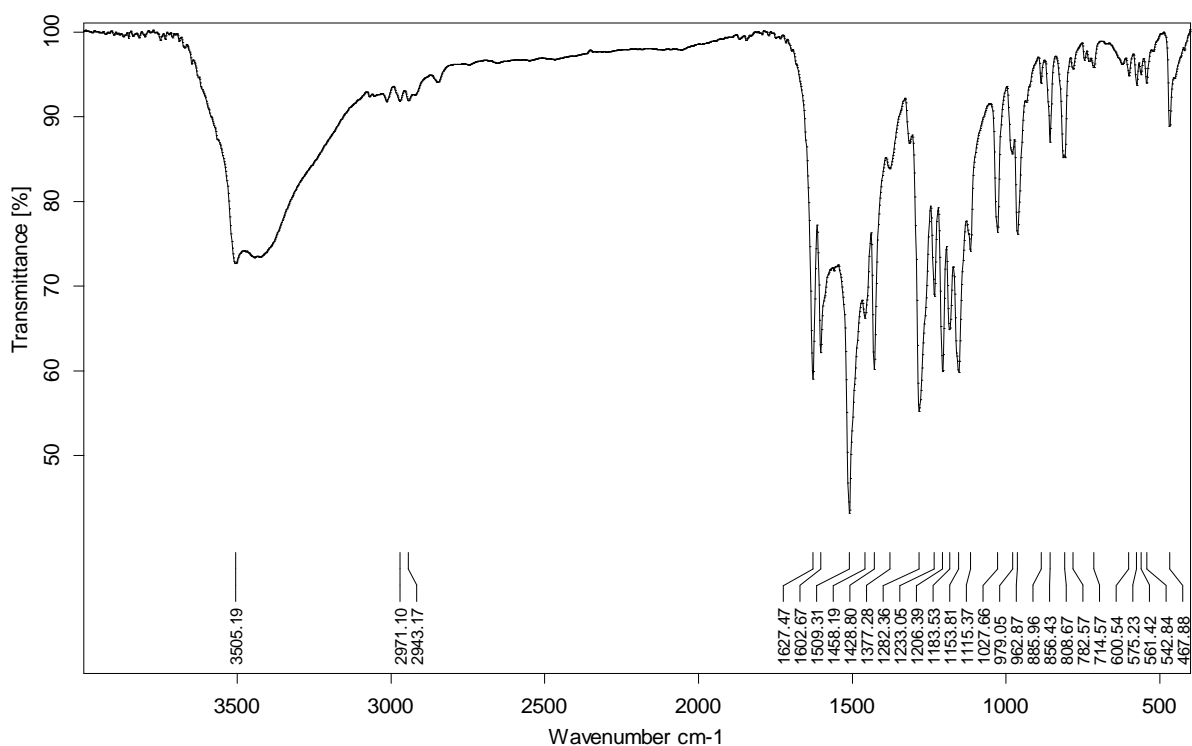
**Figure 3:** Absorption of spectra of CUR and CUR-Cu(II)

### 3.2 pH optimization of the complex formation

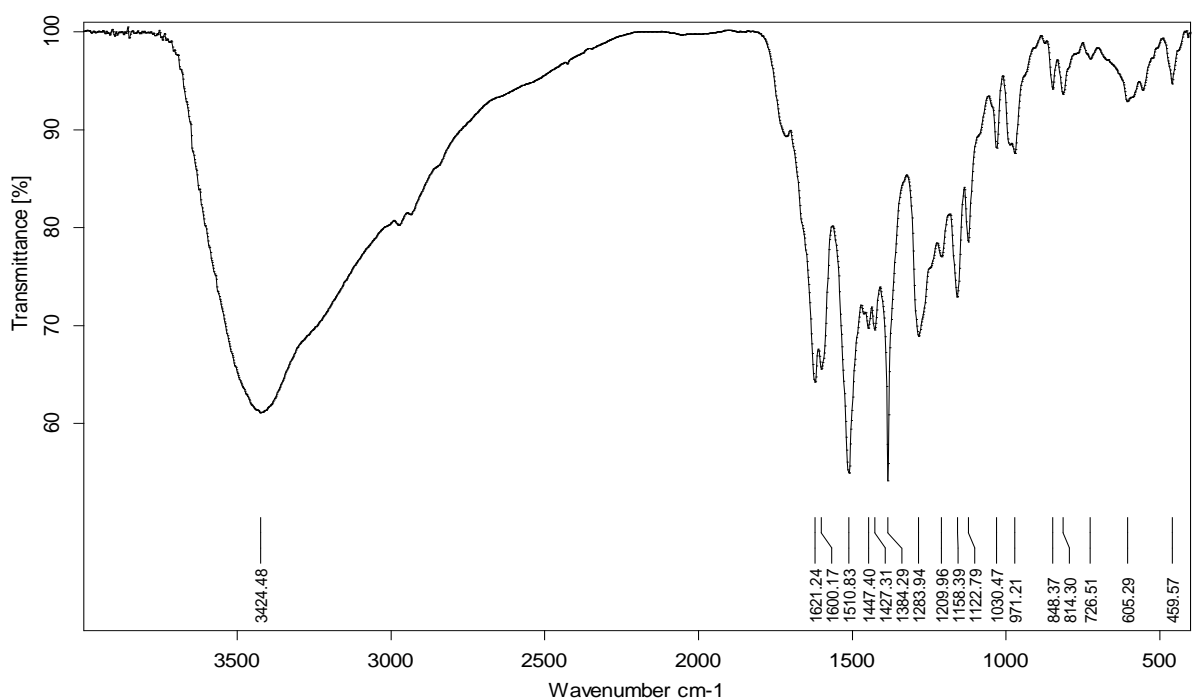
The absorption spectra of Cur complexes were obtained in the pH range 3–10. To maintain optimum conditions, appropriate concentrations of reagent Cur ( $2.0 \cdot 10^{-5} \text{ mol.L}^{-1}$ ) and M ( $2.0 \cdot 10^{-5} \text{ mol.L}^{-1}$ ) were chosen. The effect of pH on the absorbance of CUR-Fe(III) at 510 nm. The maximum of CUR-Fe(III) complex was obtained in the pH range 6–8, Therefore, the pH value of 7 was chosen for subsequent experiments. Different pH values were obtained by varying the relative amounts of NaAc and HAc, and confirmed by a digital pH-meter. While Cur-Cu (II) complex was also formed at pH = 7.5 and had a maximum absorbance at 505 nm. Cur-Zn (II) complex was formed at pH = 5.6 with the maximum absorption wavelength at 428 nm.

### 3.3 IR spectra of curcumin and its complexes

The infrared spectrum of CUR and its complexes were investigated. From results on Table 1, the Figure 4, 5, 6 and 7, the structure of curcumin was confirmed by the appearance of a weak band within the range  $3500 \text{ cm}^{-1}$  corresponding to -OH. The FT-IR spectra also showed a weak band or shoulder located at  $3051 \text{ cm}^{-1}$  which was assigned to aromatic C-H. The oscillation of the group C = O was confirmed by the frequency at stretching vibration at  $1605 \text{ cm}^{-1}$ . Compared with the reference spectrum of curcumin, all complexes showed a great decrease in the intensity of (C=O) carbonyl band, In addition, a net decrease in the intensity of the free (OH) hydroxyl group of curcumin was observed in the case of curcumin complexes. These above phenomena indicated that some interactions metal ions with functional groups.

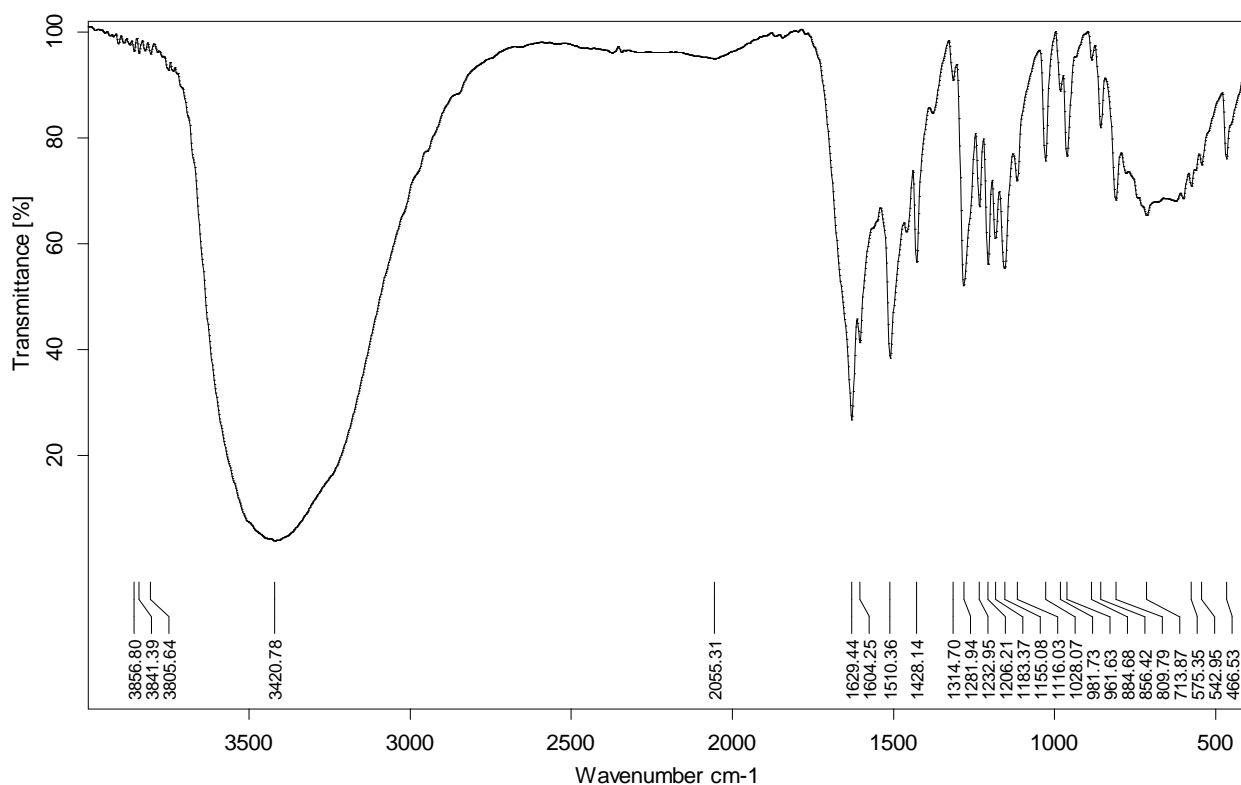


**Figure 4:** FT-IR spectra of CUR with KBr pellet

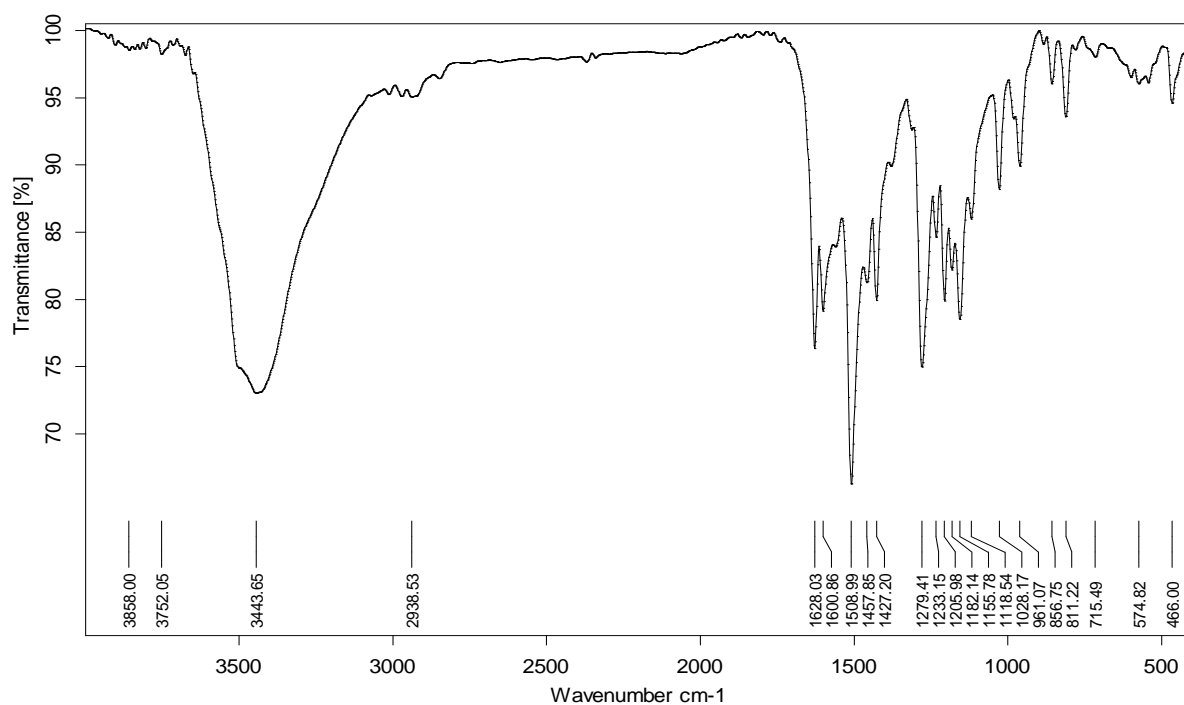


**Figure 5:** FT-IR spectra of CUR-Fe(III) with KBr pellet



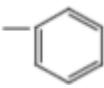


**Figure 6:** FT-IR spectra of CUR-Zn(II) with KBr pellet



**Figure 7:** FT-IR of CUR-Cu(II) with KBr pellet

**Table 1:** Wave length changes of main vibration modes from infrared (KBr pellets) spectral data of curcumin and curcumin complexes

Bonds and functional groups	Curcumin (cm <sup>-1</sup> )	Curcumin-Cu (cm <sup>-1</sup> )	Curcumin-Fe (cm <sup>-1</sup> )	Curcumin-Zn (cm <sup>-1</sup> )
-OH	3500,67	-	-	3532,40
-C=O	1626,35	1589,17	1619,85	1624,09
-C=C-	1601,87	1557,49	1589,05	1594,06
-C-O-C-	1113,59	1124,27	1121,05	1120,57
	2970,78	2971,65	2988,17	2942,10
	1505,77	1479,60	1486,93	1491,92
	1455,92	1448,65	1447,31	1451,02

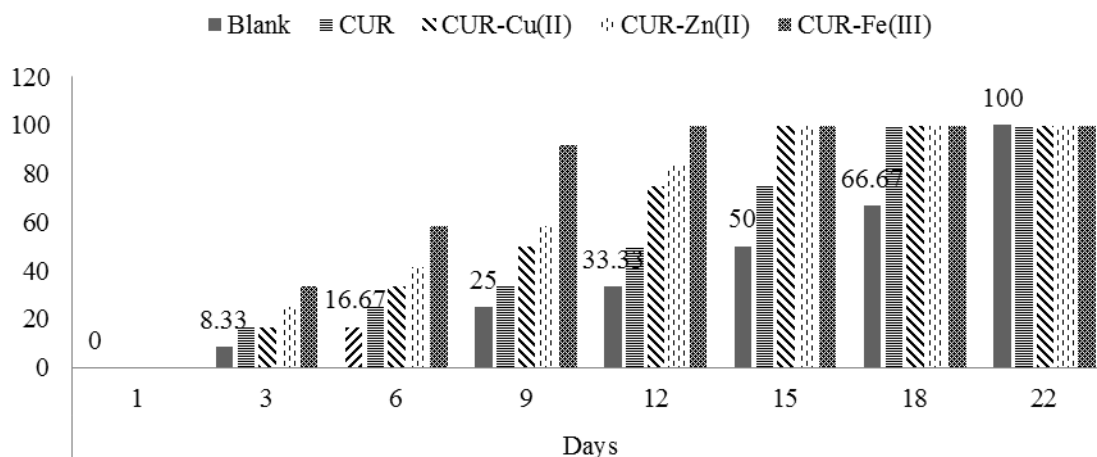
### 3.5 Initial applied in the treatment of burns in mice

In this experiment, white mice *Rattus norvegicus* provided by the Drug Testing Center of the University of Medicine HCM City were selected for studying. Experimental mice were commonly used in scientific experiments in the fields of medicine, biology, psychology or other fields. The test mice had the same weight, age, diet and living conditions. For carrying out these experiments, the mice were cut the hairs on the back and burns on the skin were made of hot coal or boiling water with the same size according to Mehrabani D method [12]. Then, each wound was applied CUR solution 3 times daily (per 10 ml). Complexes were also conducted similar methods. Reconstruction of wound on rat skin were recorded and presented in the table 2 and figure 8.

For mice not treated with CUR and complex, it was necessary 22 days to recover the wound. It took 18 days to restore with mice treated by CUR. While the restorations were equivalent to 15 days with Cur-Cu (II) and Cur-Zn (II). Specifically, the mice were treated with CUR-Fe, it took only 12 days to recover completely.

**Table 2:** The percentage of wound healing (%)

	Days							
	1	3	6	9	12	15	18	22
Blank	0,00	8,33	16,67	25,00	33,33	50,00	66,67	100,00
CUR	0,00	16,67	25,00	33,33	50,00	75,00	100,00	100,00
CUR-Cu(II)	0,00	16,67	33,33	50,00	75,00	100,00	100,00	100,00
CUR-Zn(II)	0,00	25,00	41,67	58,33	83,33	100,00	100,00	100,00
CUR-Fe(III)	0,00	33,33	58,33	91,67	100,00	100,00	100,00	100,00



**Figure 8:** The percentage of healing of burn by curcumin and its treatment

## 4 CONCLUSIONS

After study on the complexation of Curcumin with metal cations, the results are shown that Cur-Fe (III) complex is formed at pH = 7.5 at ratio of 1:1 with the maximum absorption wavelength at 510 nm. While at pH = 7.5 Cur-Cu (II) is also formed at a ratio 1:1 and has a peak at 505 nm and a 1:1 complex between of Cur and Zn (II) at pH = 5.6 with peak at 428 nm was formed. Curcumin and curcumin-metal complexes can heal burns faster than pure curcumin. The cur-Fe (III) complex is the fastest; the Cur-Cu (II) complex and the Cur-Zn (II) complex are the same time, but the Cur-Zn (II) is faster.

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## **USAGE OF ISOMALT AS BINDING AGENT FOR IMPROVING TABLETCITY OF YOGURT TABLET**

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### **ABSTRACT**

The aim of this project was to develop a low caloric and prebiotic yogurt tablet. Yogurt powder was first produced using freeze-dried method that can keep in room temperature with full of original flavor and nutrients. Also, the product can be further pressed into tablet, easy to consume and benefit to health. To improve prebiotics substances, fructo-oligosaccharide or FOS was added prior the fermentation. The addition of FOS was not only provided prebiotics property to the yogurt but also maintained the yogurt curd firmness with reducing syneresis values of developed yogurt. Prepared yogurt was then freeze-dried for 28-30 hours from  $-30^{\circ}\text{C}$  to  $32\pm 3^{\circ}\text{C}$  to obtain yogurt powder at 5-6% moisture content. The yogurt powder produced no significant difference on flowability between with and without FOS. The addition of FOS, however, induced  $b^*$  values. To optimize sweetness of prebiotics yogurt powder, three different concentrations of icing (15%, 20% and 25%) were used. As a result, 20% of sugar icing gave the highest preferences ( $7.8\pm 0.9$  out of 9). Further, four sweetener mixtures as cello-oligosaccharide (or cellobiose) mixed with enzyme modified steviol glycosides (Stevia) or sucralose and the mixture of isomaltulose mixed with Stevia or sucralose were used to substitute 20% sugar icing and compared. As results, cellobiose mixed with sucralose or Stevia gave no significant differences on liking score of color, aroma, flavor, sweetness, sourness and overall liking compared to those containing 20% icing ( $p>0.05$ ). The sample containing stevia was selected to further mix because the highest preference was obtained. The tablet pressing with binding agents, isomalt (1% and 10%) was then investigated. Higher percentage of isomalt gave better hardness for both formulations but formulation contained isomaltulose provided higher hardness than formulation containing cellobiose for both concentrations. As the sensorial properties were done, there were no significant difference in any attributes ( $p>0.05$ ). The formulation of isomaltulose with stevia and 1% isomalt was selected. In summarize, the addition of prebiotics (FOS and cellobiose) and the selected sweeteners have not altered the major characteristics of the yogurt as well as showed similar preferences to the control sample containing sugar icing. For consumer test ( $n=100$ ), the low caloric and prebiotic yogurt tablet was successfully developed as a new kind of product in the dairy field from like slightly to like moderately of overall liking ( $6.8\pm 1.5$ ).

**Keywords:** Yogurt, Tablet, Sweetener, Prebiotics, Freeze-drying, Isomalt

## 1 INTRODUCTION

Yogurt is a cultured milk product that is soured and thickened by the action of specific starter cultures, i.e. *Lactobacillus bulgaricus* and *Streptococcus thermophiles* which was added to milk to coagulate the milk protein (Dairy Council of California©, 2017). In 2016, Euromonitor International reported that yogurt products showed a strong performance, particularly in Thailand, according with health-conscious trends driving the growth of yogurt market across the globe (Future Market Insights, 2018). There were also more variants introduced, from flavors to fruits in yoghurt, to attract consumer and generate their interests. Nonetheless, yogurt has to store under proper conditions which is refrigerator temperature around 35-40°F or 2-4.4°C (White, 2008) with its best in the perior of first few weeks of shelf life, after which there is a discernible reduction in sensory characteristics (MacBean, 2009). Therefore, the attempt to produce yogurt powder which further pressed to be tablet would provide a shelf stable yogurt to introduce a new form of yogurt to the market.

In order to maintain original sensorial quality of yogurt powder with high nutritious retention, a freeze-drying is introduced. In freeze-drying process, the product is dried under sublimation of ice under pressure. It is especially useful for the product that is heat sensitive in order to keep the volatile compounds responsible for aroma and flavor (Barbosa-C´anovas, 2005). However, from the previous study (Jaya, 2009) showed that freeze-drying effects on stability of hydrophobic bonds causes more hygroscopic nature of the freeze-dried yogurt. Nevertheless, freeze dried samples produce denser protein network, which is difficult to breakdown and it has completely different structure than other types of drying which are air drying, vacuum drying and microwave vacuum drying. However, the effects of hydrogen bonds involved in binding the surface layer of protein to the cell wall of yogurt bacteria that might cause structural collapse to the yogurt product.

To produce a low calorie under a natural approach concept of product, groups of natural sweeteners were introduced to the yogurt product. In this research, fructooligosaccharide, stevia, sucralose, isomaltulose were mixed and used as sugar substitute. The addition of fructooliogosaccharide (FOS) was not only provided prebiotics property to the yogurt but also provided sweet taste as a fast release sweetener to the yogurt powder (Sabater-Molina M. et.al., 2009). Furthermore, it was found in our previous study that FOS can maintain the yogurt curd firmness with reducing syneresis values of developed yogurt (Tangsuk and Tipvarakarnkoon, 2018). To further increase an easy consumable possibility, yogurt was then pressed in a tablet form. Another sweetener, isomalt, is introduced to improve a tabletcity of the final product. A study on the characterization and direct compression properties of isomalt were reported by (Ndindayino *et al.*, 1999). Considered as an ideal sugar-free and low-calorie alternative, isomalt is similar in sweetness of sugar but without the caloric or physical risks. Isomalt is considered as an anticaking and bulking agent and can also be used in multivitamin and mineral supplements (Gillco Ingredients, 2017). Moreover, isomalt shows very low water absorption enables the

development of high shelf life sugar-free confections and also tastes quality (Sentko and Willibald-Ettle, 2012).

Therefore, the aim of this project was to develop the yogurt tablet using freeze-dried method which would provide a shelf stable, low-caloric yogurt powder with prebiotics. Also, the product can be further pressed into tablet and easily consume.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

The experimental materials consist of full fat pasteurized milk (Meji brand, Thailand), yogurt starter culture (yolida® brand, Thailand), fructo-oligosaccharides, stevia powder, isomaltulose powder, sucralose powder, isomalt powder and cellobiose powder which were kindly donated from Rajburi Sugar Co., Ltd., Thailand.

### **2.2 Yogurt powder production**

Milk was pre-heated at 60-65°C for 10 minutes prior to filter milk foam and denatured milk protein. Warm milk was homogenized at 17,500 rpm for 3 minutes then pasteurized at 80-85 °C for 30 minutes. The milk was immediately cooled down to 42-45°C and poured 300 ml milk into a 400 ml tall beaker. Heated milk was added 0% (control) and 10% w/v of fructo-oligosaccharides and inoculated with 3%w/v of commercial yogurt starter culture. To obtain stable yogurt curd, it was incubated at 45°C for 4 hours and left it cool in refrigerator (4 °C) overnight. The set yogurt was then gently stirred in order to be able to pour into the freeze-dried tray. Freeze drying method was then performed under freeze dry condition of -35 °C to 40 °C about 23-25 hours. The yogurt powder was kept in PET/PE air tight package.

### **2.3 Effect of sugar icing concentrations on sweetness of yogurt powder**

Three sets for sweetened yogurt powder were prepared by mixing the powder with sugar icing at various concentrations (15, 20, 25 g per 100 g of yogurt powder). The dry mix powder was kept in air tight package before further measurement of sensory test.

### **2.4 The optimization of sweet taste of yogurt powder using sweetener mixtures**

The optimized sugar icing concentration was selected from 2.2.2. Four different sweetener mixtures were used to replace 20% sugar icing. There were four combinations which were cellobiose-stevia, cellobiose-sucralose, isomaltulose-stevia and isomaltulose-sucralose compared with control (20% sugar icing). The sweetener was dry mixed by 50:50 or 10% of each.



## **2.5 Effect of isomalt concentrations on binding property of yogurt tablet**

The optimized sweetener mixtures were obtained from 2.2.3. The sweetened yogurt powders were mixed with 2 different concentrations of isomalt (1% and 10%). The powder was dry mixed before tablet pressing using rotary tablet pressing machine to get yogurt tablet which weighed for 0.38 gram per tablet.

## **2.6 Analytical methods**

### *2.6.1 Total solid and moisture content determination*

The moisture content of yogurt powder was determined according to the Association of Official Analytical Chemists methods (AOAC 990.20, 2016). The samples were dried in an oven at  $100 \pm 1^\circ\text{C}$  for 5 hours. Percentage of moisture content (%MC) and percentage of total solid (%TS) were calculated.

### *2.6.2 Color determination*

The color values of yogurt powder were measured using Miniscan EZ-4500L spectrophotometer (Hunter Lab co. Ltd, USA) and results were expressed in accordance with CIE Lab\* System. The measurement was done using diffuse illumination of 45/0 viewing geometry with medium large viewing area port. All measurements were done using D65/10° as a light source. L\* (Lightness; 0 = dark and 100 = bright), a\* (+ = red and - = green), b\* (+ = yellow and - = blue) values were determined at least 5 replications.

### *2.6.3 Carr's compressibility index determination*

A gram of yogurt powder was weighed and poured into 10 ml cylinder and recorded the bulk volume to calculate bulk density. Then tapped the powder for 100 times and recorded the tapped volume to calculate tapped density. Further calculated for % Carr's compressibility index and used to relate to the flow ability of the powder. Less than 18% Carr's compressibility index described as good flowability, 19 – 40% Carr's compressibility index described as fair to poor flowability and higher than 40% Carr's compressibility index described as extremely poor flowability (Moghbel and Abbaspour, 2013).

### *2.6.4 Texture analysis*

The texture of yogurt tablet samples was tested using Texture analyzer (TA-XTplus, Stable Micro System, London, UK) with 5 mm ball probe (P/5S) under condition of return distance of 20 mm, return speed of 10 mm/s, contact force of 15 g and test speed of 0.5 mm/s. At least 10 replications were done. The results were expressed in term of hardness (g) using peak force.

### **2.6.5 Sensory evaluation**

The sweetened yogurt powder was evaluated by 30 untrained panelists who are staffs or students of Assumption University using a scoring test on a 9-point hedonic scale. Samples were randomly served with 3-digit code. There was drinking water used as palate cleansing. The sensory evaluation of yogurt powder which was sweetened by 3 different concentrations of sugar icing and 4 different combinations of sweeteners were tested and compared to control (sugar icing). The liking scores were evaluated for 6 attributes which were color, aroma, flavor, sweetness, sourness and overall liking. While the sensory evaluation of yogurt tablet which had 4 different formulations of sweeteners and concentrations of isomalt was conducted for 8 attributes which were yogurt aroma, yogurt flavor, sweetness, sourness, hardness, adhere to teeth, mouthfeel and overall liking also using 9-point hedonic scale.

### **2.6.6 Consumer test**

Central location consumer test was conducted. 100 consumers at Assumption University were given a questionnaire and sample of finished product containing mixture of isomaltulose with stevia and 1% isomalt, which was packed in vacuum seal aluminum foil type package under air-tight seal condition. The questionnaire composed of three parts; consumer's behavior, product concept and preference test and later their demographic information.

## **2.7 Statistics analysis**

Analysis of variance (ANOVA) with Duncan's Multiple Range Tests (DMRT) were used to analyze the data. Data analysis was done using R-program version 3.4.0 (R Core Team, 2017). All frequency datas from consumer test were analyzed using Pivot Excel Table version 2016.

## **3 RESULTS AND DISCUSSION**

### **3.1 Effect of FOS on yogurt powder production**

In order to develop yogurt powder, fructooligosaccharide (FOS) was added prior yogurt curd formation. On our previous study, the addition of FOS showed a positive effect on yogurt curd. The syneresis of set yogurt was reduced and accordingly induced higher percentage of yield in set yogurt (Tangsuk and Tipvarakarnkoon, 2018). In this research, therefore, also applied 10% FOS prior 5 hours incubation. The effect of FOS was then further investigated on the physicochemical properties of yogurt powder. The results were then compared to a control sample (0% FOS). As shown in Table 1, yogurt powder with the addition of FOS significantly increased percentage of yield ( $\alpha = 0.05$ ). Also, the addition of FOS showed higher moisture content in yogurt powder. It implied that FOS would help retention of water content in dried powder. The fact that FOS is oligosaccharide composed of linear chains of fructose units (Sabater-Molina *et al.*, 2009). The presence of FOS in yogurt would increase the amount of monosaccharide content that help trapping more water in yogurt curd and further in dried powder. As results, although the addition

of FOS increased percentage of moisture content, but the effect was slightly (1.13%) compared to the significantly increase in yield percentage (5.6%).

Carr's compressibility index was calculated from bulk and tapped density of the powder which related to flow ability of yogurt powder. Typically, the value of Carr's compressibility index should lower than 40% (Moghbel and Abbaspour, 2013). The value found in the product in this research fall into a range of value that expressed as extremely poor flow ability. However, the results have indicated that FOS showed no effect in total solid and flow ability of the powder. For color, sample containing FOS showed higher L\* and b\* value, but the effect was slightly.

**Table 1:** Percentage of yield and physicochemical properties of yogurt powder

Yogurt powder	Yield* (%)	Moisture content* (%)	Total solid <sup>ns</sup> (%)	Carr's compressibility <sup>ns</sup> index (%)	L*( <sup>ns</sup> )	a*(*)	b*(*)
0% FOS	12.50	5.43±0.19	94.57±0.19	79.68± 6.43	90.95 ± 0.01	-0.55 ± 0.04	10.26 ± 0.01
10% FOS	18.10	6.56±0.37	93.44±0.36	79.66± 4.65	93.38 ± 0.01	-0.16 ± 0.01	11.34 ± 0.17

Remark: ns- no significant difference

\*- significant difference at  $p < 0.05$

### 3.2 Effect of icing concentrations on sweetness of yogurt powder

According to the positive effect of FOS, the yogurt powder in the presence of FOS was produced and further sweetened by mixing with three different concentrations (15, 20, 25%) of sugar icing. As results (Table 2), there were no significant different in any sensory attributes ( $p > 0.05$ ) except sourness. Higher amount of sugar would reduce the spike of sourness when consume that could be more preferred in sourness attributes. Sample containing 20% sugar icing was chosen for further study due to the most selected about 46% from 30 panelists.

**Table 2:** Mean liking scores of yogurt powder at different icing concentrations (n=30) and percentage of most preferred sample chosen by panelists

Sugar icing (%)	Color <sup>ns</sup>	Aroma <sup>ns</sup>	Flavor <sup>ns</sup>	Sweetness <sup>ns</sup>	Sourness	Overall liking <sup>ns</sup>	Most preferred
15	7.9±1.0	7.1±1.0	7.3±1.0	7.0±1.1	7.0±1.3 <sup>b</sup>	7.4±1.0	13.3%
20	7.7±1.2	7.3±0.9	7.6±0.9	7.5±1.0	7.6±1.2 <sup>ab</sup>	7.8±0.9	46.7%
25	7.9±0.9	7.3±1.1	7.5±0.9	7.5±1.0	7.6±1.1 <sup>a</sup>	7.7±1.0	40.0%

Remark: ns- no significant difference

a,b,ab - no significant difference with the same letters within the same column ( $P > 0.05$ )

### 3.3 Optimization of sweet taste for yogurt powder using sweetener mix

Results from the previous section showed that 20% sugar icing showed highest preference. In order to replace sugar and their calories, sweeteners were used. The sweeteners used in this study include cellobiose, isomaltulose, stevia and sucralose which had different relative sweetness of 0.1, 0.5, 300 and 600, respectively. The relative sweetness of sucrose equal to 1. Four combinations of sweeteners were used according to the mixing between low intensity sweeteners (cellobiose and isomaltulose) and high intensity sweeteners (stevia and sucralose). As shown in Table 3, the mixture of sweeteners had not affected on the mean liking scores of color and aroma of yogurt powder. Among them, samples containing isomaltulose-sucralose obtained lowest liking score in flavor, sweetness, sourness and overall liking. Stevia was then selected for further tablet pressing due to high overall liking ( $7.2 \pm 1.0$  and  $7.1 \pm 1.1$ ) and close to control ( $7.5 \pm 1.0$ ). It also benefits to gain more natural substances in the product.

**Table 3:** Mean liking score of yogurt powder containing different sweeteners (n=30)

Sweeteners (50:50)	Color <sup>ns</sup>	Aroma <sup>ns</sup>	Flavor	Sweetness	Sourness	Overall
<b>Cellobiose : Stevia</b>	$7.7 \pm 1.0$	$6.8 \pm 1.2$	$7.3 \pm 1.0^a$	$6.9 \pm 1.3^{ab}$	$6.9 \pm 1.5^a$	$7.2 \pm 1.0^a$
<b>Cellobiose : Sucralose</b>	$7.8 \pm 0.8$	$6.8 \pm 1.0$	$7.1 \pm 1.1^{ab}$	$6.9 \pm 1.3^{ab}$	$6.8 \pm 1.3^a$	$7.3 \pm 1.0^a$
<b>Isomaltulose : Stevia</b>	$7.5 \pm 0.9$	$7.1 \pm 1.1$	$7.0 \pm 1.1^{ab}$	$6.9 \pm 1.3^{ab}$	$7.2 \pm 1.0^a$	$7.1 \pm 1.1^{ab}$
<b>Isomaltulose : Sucralose</b>	$7.7 \pm 1.1$	$6.8 \pm 1.3$	$6.6 \pm 1.2^b$	$6.3 \pm 1.4^b$	$6.1 \pm 1.5^b$	$6.6 \pm 1.0^b$
<b>Control (20% icing)</b>	$7.7 \pm 0.9$	$7.1 \pm 1.2$	$7.4 \pm 1.1^a$	$7.1 \pm 1.3^a$	$7.2 \pm 1.1^a$	$7.5 \pm 1.0^a$

Remark: ns- no significant difference

<sup>a,b,ab</sup> - no significant difference with the same letters within the same column ( $P > 0.05$ )

### 3.4 Effect of isomalt concentrations on binding property of yogurt tablet

Isomalt is one of well-known sweetener used in powder and confectionary product due to its distinctive of tablecity and hygroscopic property. The aim of this experiment was to investigate the effect of isomalt onto texture of yogurt tablet. Isomalt was selected to use as a binding agent for improving yogurt tablecity in product. Two concentrations of isomalt (1 and 10 g per 100 g of sweetened yogurt powder) were mixed with two different combinations of sweeteners which were cellobiose-stevia and isomaltulose-stevia at a ratio of 50:50. As results (Table 4), the increment in the concentration of incorporated isomalt resulted in harder texture of yogurt tablet for both samples. These results agreed with Pourmohammadi *et al.* (2017) which was also found that increasing in concentrations of isomalt increased hardness of biscuit products. It also showed that the formulation contained isomaltulose gave harder texture than those containing cellobiose for both concentrations of isomalt.

**Table 4:** Hardness (g) and coefficient of variation (C.V.) of yogurt tablet in different concentrations of isomalt

Tablet mixture	Yogurt Aroma <sup>ns</sup>	Yogurt Flavor <sup>ns</sup>	Sweet <sup>ns</sup>	Sour <sup>ns</sup>	Hardness <sup>ns</sup>	Adhere to teeth <sup>ns</sup>	Mouthfeel <sup>ns</sup>	Overall liking <sup>ns</sup>
<b>Cellobiose with stevia</b>								
1% isomalt	6.8±1.6	6.9±1.1	6.8±1.0	7.0±1.2	6.9±1.5	6.5±1.5	6.9±1.0	7.1±1.0
10% isomalt	6.4±1.5	6.5±1.1	6.6±1.0	6.8±1.0	6.6±1.4	6.6±1.1	6.6±0.9	6.6±0.9
<b>Isomaltulose with stevia</b>								
1% isomalt	6.1±1.5	6.5±1.1	6.5±1.3	6.7±1.1	6.9±1.6	6.7±1.3	6.9±0.9	6.9±1.0
10% isomalt	6.2±1.6	6.5±1.5	6.5±1.0	6.8±1.0	6.5±1.6	6.1±1.3	6.6±1.2	6.6±1.2

**Table 5:** Mean liking scores of yogurt tablet at different mixtures of sweeteners (n=30)

Test composition		Hardness (g force)	
		Average	C.V.
<b>Cellobiose with stevia</b>	<b>1% isomalt</b>	2380.21	57.61
	<b>10% isomalt</b>	4836.09	17.56
<b>Isomaltulose with stevia</b>	<b>1% isomalt</b>	4878.14	35.57
	<b>10% isomalt</b>	7184.40	67.42

Remark: ns - no significant difference

<sup>a,b,ab</sup> - no significant difference with the same letters within the same column ( $P > 0.05$ )

The effect of isomalt was also investigated on the preference of the yogurt tablet product. As shown (Table 5), there was no significant difference in any sensory attributes ( $p > 0.05$ ). Even sample containing 10% isomalt showed higher hardness value (Table 4), however, it was not affected to the liking of the products. For further study, the combination of isomaltulose and stevia was selected to use in consumer test due to its cheaper cost and higher market availability than cellobiose.

**Table 6:** Demographic information developed product preference and attitudes toward the developed product (n=100)

Question/choices		Percentages		
		female	male	Total
<b>1. Gender</b>		<b>62</b>	<b>38</b>	<b>100</b>
<b>2. Ages</b>				
	12-18 years	8	21	13
	19-24 years	76	76	76

Question/choices	Percentages		
	female	male	Total
> 25 years	15	3	11
<b>3. How would you rate this product?</b>			
(Liking scores out of 9)	6.8±2.1	6.9±2.7	6.8±1.5
<b>4. If product has 25 tablets per pack, how much will you be willing to pay?</b>			
15-20 baht	48	61	53
21-25 baht	35	24	31
26-30 baht	11	16	13
more than 30 baht	5	0	3
<b>5. For whom will you purchase this product for?</b>			
Yourself	76	58	69
Sharing with group of friends	18	24	20
For whole family together enjoy	3	13	7
Your children	3	5	4
<b>6. What are factors that you will consider if you purchase this yogurt tablet?</b>			
Health benefit	2	5	3
New product	6	5	6
Nutrition	3	3	3
Price	66	63	65
Shop location	0	1	1
Taste/flavor	23	22	22
<b>7. Which type of packaging do you prefer for this product?</b>			
Aluminum foil bag	27	24	26
Glass jar	52	61	55
Plastic box	8	5	8
Transparent plastic bag	11	11	11
<b>8. Level of agreement on the statements (from 1 = extremely disagree, 5 = extremely agree)</b>			
(a) I want to enjoy it as snack	3.9±0.8	4.0±0.8	3.9±0.8
(b) It would help my digestive system	3.7±0.7	3.8±0.8	3.8±0.8
(c) It is too sweet	2.9±0.7	2.8±0.9	2.8±0.9
(d) It would make me fat	2.7±0.7	2.7±0.9	2.7±0.8
(e) It is a healthier food choice	3.7±0.8	3.9±0.8	3.8±0.8

Question/choices	Percentages		
	female	male	Total
(f) It differs from product in current market	3.8±1.0	4.1±0.7	4.0±0.8
(g) It is good for everyone	3.9±1.0	4.2±0.7	4.1±0.8
(h) I will eat it only when I am stressed	2.9±0.9	3.0±1.2	3.0±1.1

### 3.5 Consumer test

100 consumers were asked to test the yogurt tablet product (isomaltulose-stevia and 1% isomalt) developed in this experiment. The results of product preference and attitudes toward product have been reported in Table 6. As results, 62% of female and 38% of male consumer with age ranged at highest frequency of 19 – 24 years old tested the products. Studies showed that more than 60% of them consumed yogurt regularly every week. As product testing, overall liking score were like slightly to moderately like (6.8±1.5 out of 9). Similar results have been found in both male and female consumer. Consumer were agreed to pay less than 30 baths per pack (25 tablets). They were willing to buy the product for themselves (69%) or sharing with groups of friends (20%) instead of family sharing. Once they were asked about the factors that would affect most to the purchasing decision, price and taste were the most important factor among all. They preferred to pack the product in glass jar rather than others. On the attitude toward products (score out of 5.0), they somewhat agreed that this product is good for everyone (4.1), it was differed from other products in the market (4.0), can be enjoy as snack (3.9) and considered as healthier choice with could help digestive system (3.8).

## 4 CONCLUSIONS

In conclusion, the addition of prebiotics (FOS and cellobiose) and sweeteners showed similar preferences of the developed yogurt powder to the control sample containing sugar icing meanwhile have not altered the major physicochemical characteristics of the yogurt powder. Addition of FOS helps improving yield percentage in yogurt powder despite increasing slightly its moisture content. The best combination formulation found from this research was isomaltulose with stevia (50:50) and 1% isomalt. The addition of isomalt improved binding capacity of yogurt tablet as shown in improving hardness of tablet. For consumer test, this project is successfully developed a new form of product in the dairy field from like slightly to like moderately of overall liking (6.8±1.5).

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## **STUDY OF EFFECT OF MIXED EMULSIFIERS ON STABILITY OF VIRGIN COCONUT OIL (VCO) EMULSION ENRICHED WITH HYALURONIC ACID**

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### **ABSTRACT**

Formulation, characteristic, and stability of the virgin coconut oil (VCO) emulsion enriched with hyaluronic acid (HA) were studied. As VCO contains high phenolic compounds it exhibits antioxidant activity which gives many benefits for human health. HA is heteropolysaccharide and natural substance in skin that moisturiz and replenish skin to enhance the healthy look.

VCO emulsion was performed by using mixed emulsifiers of Tween 20 and Span 80 to achieve the HLB value of 4, 5, 6, 7, 8, 9 and 10; the ratio of water (W) and mixed emulsifiers (E) was 1:5 and the ratio of [W:E] and VCO (O) were 1:1, 1:2, 1:3, and 1:4, respectively. This preliminary study showed that the required HLB suitable for VCO emulsion were 7, 8 and 9 while the ratio of [W:E] : [O] were 1:3 and 1:4. These HLB values and ratios of [W:E] : [O] were subsequently used to optimize the appropriate ratio of W, E and O. The stability and characteristics of emulsion were observed in 8 formulas for each HLB value, then two best formulae for each HLB were selected in which water were substituted with HA in concentration. The results indicated that for value HLB 7, the stability percentage of formula F containing VCO enriched with 0.4 % w/v HA solution was highest and reached  $90.50 \pm 2.23$ . When prepared with value HLB 8, VCO emulsion enriched with 0.2 % w/v HA solution, formula E, gave the best stability value. The optimum formula for highest HA (1% w/v solution) enriched VCO emulsion is formula A with HLB 9, 1[HA:E] : 4[O], containing 3.32% w/w HA, 16.68 w/w E, and 80% VCO.

**Keyword:** Virgin coconut oil (VCO), Hyaluronic acid, Water in oil emulsion, Mixed emulsifier, HLB

### **1 INTRODUCTION**

Nowaday, emulsion is widly used in many fields such as food indutries, agrochemicals, medicinal and pharmaceutical industries and preferably in cosmetic and personal health care products due to its advantages and beneficiais. Emulsion is the mixing of at least two immisible liquids by emulsification which requires a single emulsifier or mixed emulsifiers to stabilize the homogeneous emulsion. Emulsion is refered as a heterogeneous system, consisting of dispersed phase as droplets in the continuous phase. Two basic types of emulsion are obtained as either

water in oil (w/o) emulsion, water droplets dispersed in an oil phase or oil in water (o/w) emulsion, oil droplets dispersed in an aqueous phase [1].

Emulsifiers or surfactants reduce the surface tension by position on the interface and contribute the stabilizing effect to the emulsion [2]. Nonionic emulsifier is the surfactant that has no charge at the hydrophilic portion but it is polarized and shows the property similar to charge. This nonionic emulsifier is generally recognized as being safe, biocompatible and unaffected by pH change. It is also commonly used in the production of emulsion of cosmetic products since it did not provide a charged molecule that are harmful to the skin [3, 4]. Sorbitan monooleate (Span 80) and polyethylenesorbitan monooleate (Tween 20) are examples of nonionic emulsifiers [5].

Coconut is the commercial fruit in eastern Asia countries. Many benefits are obtained from coconut as it is rich of calories, vitamins and minerals. The water part is a very good refreshing drink because it contains sugar, electrolytes, minerals and bioactive compounds that help the metabolism and digestion. The oil that obtained from kernel is a very good emollient agent used in food, hair therapy, medicine and cosmetic. Various techniques have been used to extract coconut including refined process and unrefined process. The refined process for producing coconut oil involves baking and bleaching stage which reduce the amount of polyphenols and medium chain saturated fatty acids. Unrefined coconut oil is extracted from the fresh coconut by fermentation and centrifugation. This coconut oil has not pass through the bleaching step which makes the unrefined oil also known as virgin coconut oil (VCO) [1]. VCO is widely available in Thailand in two different forms and particularly used for food products and cosmetics, personal health care and pharmaceutical products. Lauric acid, the saturated fatty acid, is the main fatty acid approximately 45.1% in VCO. Many researchs have reported that VCO have antibacterial, antiviral, antiprotozoal and antioxidant activities.

Hyaluronic acid (HA) is a polysaccharide of repeating unit of N-acetyl-D-glucosamine and glucuronic acid. HA naturally presents in almost biological fluids and tissues, especially in skins. HA shows many important roles in skin such as immobilizing due to increasing water in tissue, influence cell proliferation and differentiation as well as repairing skin tissue. HA also plays a role as a scavenger of free radicals generated by the ultraviolet rays from sunlight. According to these advantages, HA is widely used in cosmetics industry because it helps improve skin appearance, retaining collagen, increasing moisture and the elasticity and flexibility of skin [6].

This study was aim to obtain w/o emulsion containing VCO as the oil phase, hyaluronic acid as the water phase, and the mixture of Tween 20 / Span 80 as the emulsifiers. The stability of emulsion was evaluated based on the different hydrophilic-lipophilic balance values (HLB) to obtain the most suitable VCO emulsion enriched with HA.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Virgin coconut oil (VCO), brand MANATURE of Pordee Co. Ltd., which used for cold extraction by centrifuge process was purchased from the local grocery store. Hyaluronic acid (HA) or Tremella fuciformis Sporocarb polysaccharide were developed by Shanghai Huiwen Biotech Co. Ltd.. Sorbitane monooleate (Span 80) and Polyethylene glycol sorbitan monolaurate (Tween 20) from Sigma-Aldrich were used as emulsifiers.

### 2.2 Preparation of mixed emulsifiers (Span 80 : Tween 20)

Nonionic emulsifiers, Span 80 (HLB 4.3) and Tween 20 (HLB 16.7) were used to prepare the mixed emulsifiers which have different HLB values of 4, 5, 6, 7, 8, 9 and 10. The required amount of Span 80 and Tween 20 used in mixed emulsifiers is shown in table 1.

**Table 1:** The amount of Span 80 and Tween 20 used in the preparation of required HLB value

HLB	Span 80 (g)	Tween 20 (g)	Total (g)
4	100	0	100
5	97	6	100
6	86	14	100
7	78	22	100
8	70	30	100
9	62	38	100
10	54	46	100

### 2.3 Determination of HLB value of VCO emulsion

The w/o emulsion of VCO was prepared. The mixture of mineral water and mixed emulsifiers in a ratio of 1:5 (w/w) were taken in to 50 ml Erlenmeyer flask and stirred on magnetic stirrer at maximum speed, then VCO was added slowly while being stirred for 15 min. The ratios of [water: mixed emulsifier] : [VCO] were 1:1, 1:2, 1:3, and 1:4. The emulsions were transferred into tubes and leaved for 24 hrs. The suitable HLB values of mixed emulsifiers for each VCO emulsion were characterized by the presence of clear and transparent w/o emulsion. In addition, the stability of emulsion was determined according to Cho et al. (2008) [7] by measuring the height of the emulsion and separation phase, then calculating the percentage of stability, and also observing characteristics of emulsion after 24 hrs. Thus, the mixed emulsifier with the HLB value that gave clear emulsion and good stability were chosen for further experiments.

## 2.4 Determination of the water : mixed emulsifiers : VCO ratio

According to the selected HLB values which showed clear W/O emulsion, the ratio of water, mixed emulsifiers and VCO was examined. The mixture of water and mixed emulsifiers (W: E) was prepared in ratio of 1:2, 1:3, 1:4, and 1:5 (w/w). Furthermore, emulsions were prepared with the ratios of (W:E) and VCO 1:1, 1:2, 1:3 and 1:4 (w/w) as method described in section 2.3. Therefore 16 formulations of emulsion were obtained as listed in table 2.

**Table 2:** Formulation of emulsions with diferent water: mixed emulsifier: VCO ratios

Sample	Water (g)	Mixed emulsifier (g)	VCO (g)	(W:E)	(W:E):O
A	3.32	16.68	80	1:5	1:4
B	4	16	80	1:4	1:4
C	5	15	80	1:3	1:4
D	6.66	13.34	80	1:2	1:4
E	4.15	20.85	75	1:5	1:3
F	5	20	75	1:4	1:3
G	6.25	18.75	75	1:3	1:3
H	8.33	16.68	75	1:2	1:3
I	5.53	27.77	66.7	1:5	1:2
J	6.67	26.64	66.7	1:4	1:2
K	8.33	24.98	66.7	1:3	1:2
L	11.09	22.21	66.7	1:2	1:2
M	8.3	41.7	50	1:5	1:1
N	10	40	50	1:4	1:1
O	12.5	37.5	50	1:3	1:1
P	33.35	33.35	50	1:2	1:1

## 2.5 Determination of HA: mixed emulsifiers: VCO ratio

Hyaluronic acid was prepared in concentration of 0.2, 0.4, 0.6 and 0.8% w/w. The water phase was substituted by HA solution and then mixed with VCO to prepare emulsion as the previous described method.

## **2.6 Stability evaluation of VCO emulsion enriched with HA**

The stability of w/o emulsion during storage at room temperature was examined. 10g of emulsion was collected in the tube (diameter 1.5 cm) then the stability value was obtained by measuring the height of separation phase and emulsion phase every 12 hrs during 72 hrs of storage. The pH was also examined by using pH meter.

## **2.7 Microscopic study**

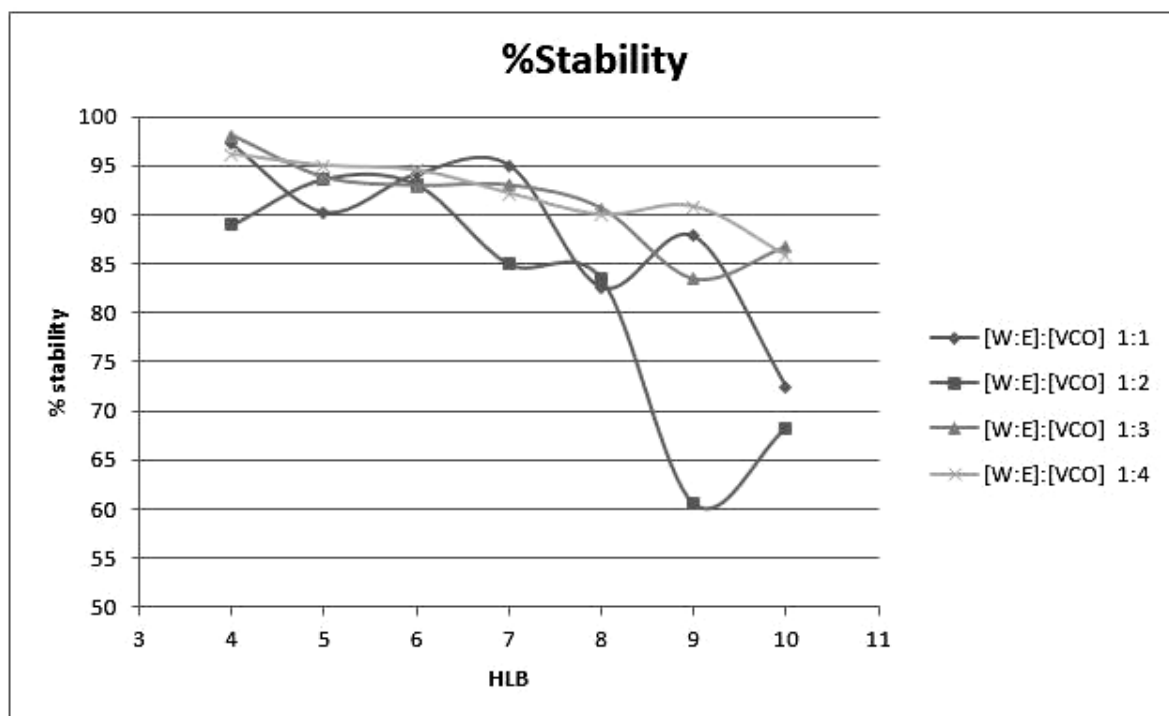
The microscopic observation was taken from the proper formulas to determine whether they were w/o or o/w emulsion. A drop of emulsion was placed on the slide and mixed with methylene blue, water soluble dye, then covered with a cover slide and observed under the light microscope. Oil in water emulsion would be indicated if the blue color surrounded the droplet. In contrast, if the blue color was at the center and outer phase was clear, it would be water in oil emulsion.

## **3 RESULTS AND DISCUSSION**

### **3.1 Influence of HLB value to the stability and the characteristic of VCO emulsion**

According to the preliminary study of determining the HLB value for w/o VCO emulsion, different amount of emulsifiers, Span 80 and Tween 20, were prepared to reach various HLB values of 4, 5, 6, 7, 8, 9, and 10. The ratio of water [W] : mixed emulsifier [E] was prepared as 1:2, 1:3, 1:4 and 1:5 and the ratio of [W:E] and [VCO] were 1:1, 1:2, 1:3, and 1:4 (w/w), respectively (Table 2). The using of water and mixed emulsifiers in the ratio of 1:5 indicated that the proper characteristics of emulsion was determined by the amount of VCO used in the emulsion. These results were similar to that of Rukimini A., et al. (2012) [8]. The transparent w/o microemulsion can only be formed when the ratio of water and emulsifier was 1: 3.5 (w/w) or 1: 4.5 (w/w).

In figure 1, the ratio of 1:1 [W:E] (w/w) showed good stability at the low HLB value of 4-7. However, the stability decreased when the HLB value was above 7, whereas the emulsion showed the white turbidity and was not clear by naked eye. For the ratio of [W:E]: [VCO] 1:2 (w/w), the highest stability was found in HLB 5 and HLB 6, and the stability decreased in HLB 7. At value HLB 9, a very clear emulsion was obtained but its stability was very low compared to others. By naked eye, emulsion with white droplet and white turbidity could be observed in most HLB values.



**Figure 1:** Percentage of stability of VCO emulsion prepared with different HLB values in the proportion of 1:5 [W:E], the ratio of [water : mixed emulsifier ]:[ VCO] were 1:1, 1:2, 1:3, and 1:4

Emulsion with the ratio of [W: E]: [VCO] 1:3 (w/w) and 1:4 (w/w) showed high stability, percentage of stability was higher than 78% for all HLB values and the emulsions were clear and transparent.

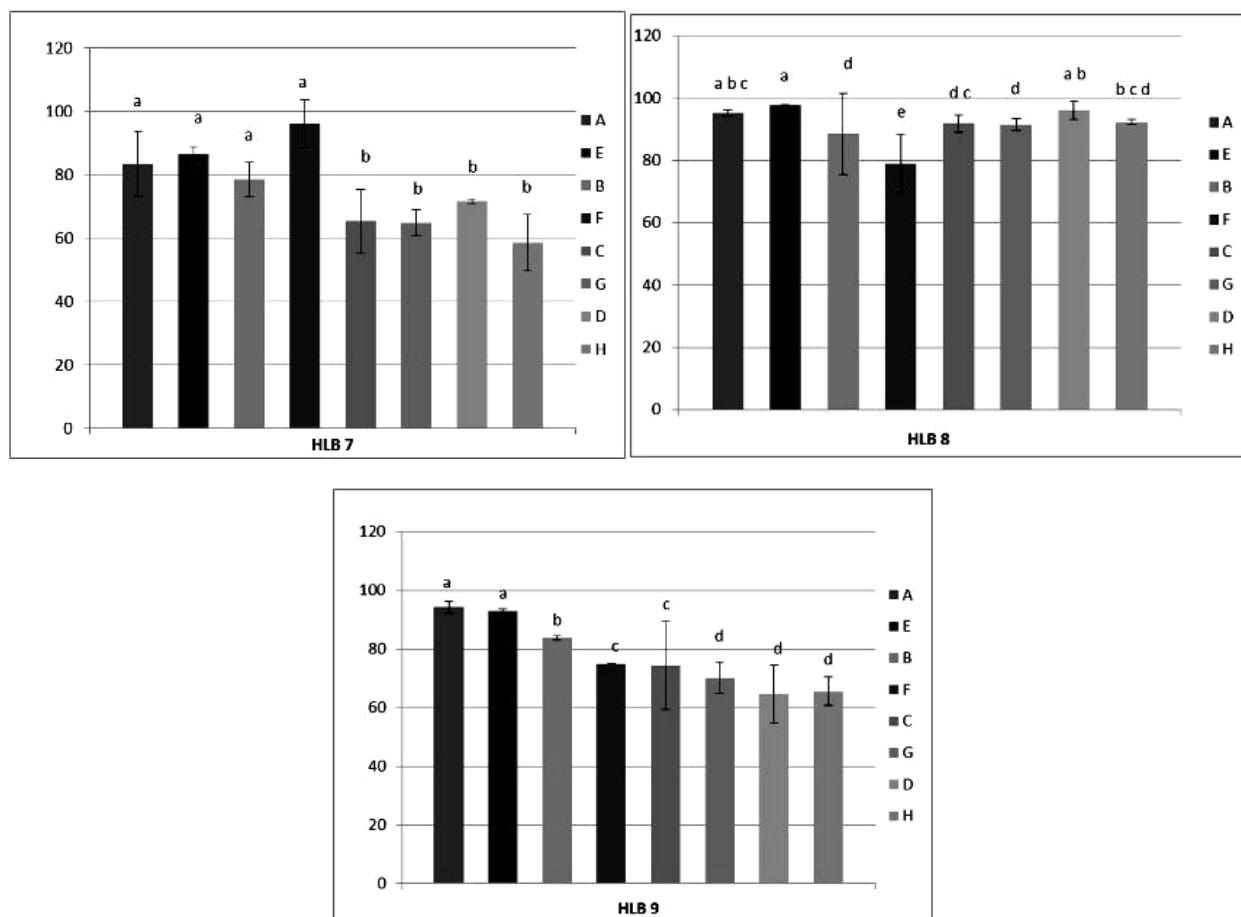
According to Rukimini et al. (2012) [8], transparent appearance and stable w/o microemulsion can be obtained by using surfactants having the HLB value of 7.0 which is close to the required HLB of VCO emulsion. So HLB values of 7, 8, and 9 were chosen for studying the stability of VCO emulsion in the further step as they showed the clear and transparent emulsion. Furthermore, ratio of [W:E]:[VCO] 1:3 and 1:4 (w/w) were also selected to study the stability of VCO emulsion enriched with HA. Another formulae could not be used to form VCO emulsion because insufficient ratio of mixed emulsifiers in the emulsion system and also unsuitable HLB value of these formulas can not provide hydrophilic and lipophilic balance to stabilize the droplet of water in VCO emulsion. According to Lawrence et al. (2012) [9] and Friberg et al. (1992) [10], the concentration of surfactants must be high enough to provide the number of surfactants to stabilize the disperse phase. Besides, mixed emulsifiers induce the interaction of the emulsifiers with water and oil, providing a more efficient way to bridge the hydrophilic-lipophilic gap between the emulsifier and water or oil than using a single emulsifier [9, 10].

### 3.2 Formulation of VCO emulsion

Eight formulae (A, B, C, D, E, F, G and H) which contain the proportion of [W:E] : [VCO] 1:3 and 1:4 (w/w) (Table 2) were prepared and used to identify the appropriate ratio showing the high value of stability. According to figure 1, mixed emulsifiers with HLB values of 7, 8 and 9 were

selected to formulate the VCO emulsion. Two formulae that showed clear transparent emulsion with the highest stability would be selected to perform the VCO emulsion enriched with HA.

As shown in figure 2, formula F and E with HLB 7 had the stability percentage of  $96.15 \pm 7.46$  and  $86.61 \pm 2.27$  respectively and showed clear transparent emulsion. Almost all VCO emulsions prepared by mixed emulsifier with HLB 8 showed high stability during storage at room temperature for 24 hrs. However only two formulae, D (stability percentage  $96.56 \pm 0.99$ ) and E (stability percentage  $97.82 \pm 0.86$ ) could give clear transparent emulsion, another formula gave the emulsion with white turbidity. For value HLB 9, formulae A and B showed highest value of stability ( $94.19 \pm 2.07$  and  $92.83 \pm 0.72$  respectively) compared to that of another formulae. As described by Cho (2008), Garti (2005) and Li (2005) [7,11,12], the combination of high and low HLB values of emulsifiers and proportion of water phase and oil phase provides the necessary conditions for the formation of a stable w/o microemulsion.



**Figure 2:** Percentage of stability of VCO emulsion with HLB 7, 8 and 9, VCO emulsions were prepared by varying the ratio of W:E into 1:2, 1:3, 1:4 and 1:5 whereas the ratio of [W:E] : [VCO] were 1:3 and 1:4. Different letters (a,b,c,d) above the columns indicate significant difference between eight groups under the same value HLB (P<0.05)



### **3.3 Enrichment of VCO emulsion by HA solution**

HA solution of 0, 0.2, 0.4, 0.6 and 0.8 % w/v were prepared and then added into VCO emulsion instead of water. The effect of HA solution to the VCO emulsion was examined by observing the characteristic of emulsion and measuring the stability percentage after storage of the VCO enriched with HA at room temperature for 72 hrs. The measurement of the phase separation was monitored for 12 hrs interval from 12 hrs until 72 hrs. Most of the VCO emulsion enriched with HA showed the trend that the higher concentration of HA solution, the lower stability of the emulsion. However as the time proceed from 12 hrs till 24 hrs, the stability of each formula was not decreased significantly.

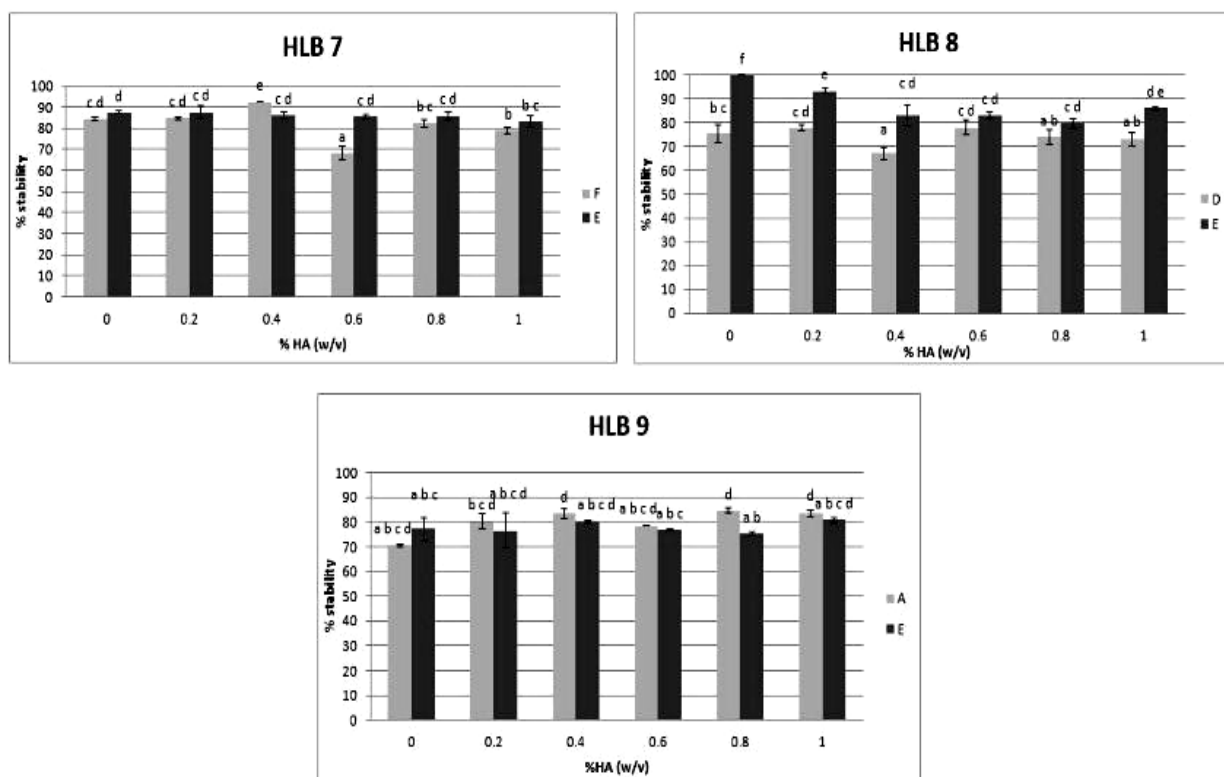
As shown in figure 3, for value HLB 7, the best formula F was obtained at HA concentration of 0.4 % (w/v) and 0.2 % (w/v) for formula E. The stability percentage of formula F containing VCO enriched with 0.4 % (w/v) HA solution was significantly higher ( $p \leq 0.05$ ) compared to that of formula E ( $90.50 \pm 2.23$  and  $88.67 \pm 0.78$  respectively).

When prepared with value HLB 8, VCO emulsion enriched with 0.2 % (w/v) HA solution, formula E, had significantly higher stability than that of VCO emulsion loaded with 0.6 % (w/v) HA solution, formula D ( $p \leq 0.05$ ). The stability percentage of formula D and formula E was  $78.12 \pm 3.12$  and  $93.28 \pm 1.05$  respectively. Therefore, formula E with 0.2% (w/v) HA was chosen as it showed higher stability.

With value HLB 9, both formulae A and E, enriched with high HA concentration and exhibited higher stability than that of formulae with HLB 7 and HLB 8. The highest stability percentage of  $83.71 \pm 1.27$  was obtained in formula A enriched with 1% (w/v) HA solution. However there was no significant difference in the stability value among different concentration of HA.

According to Wiyani et al. (2016) [5], VCO emulsion remained stable during storage if it contained surfactant:water ratio of 4.5:1 or higher in the emulsion of 77.78% oil or higher. Table 2 showed the percentage of water (or HA), mixed emulsifiers and VCO used to formulate the emulsion of selected formulae A, D, E and F with HLB 7, 8, and 9. Formula D had low stability value since it contained [W]:[E] of 1:2. Formulae A, E and F showed higher stability value because in these emulsions, ratio of water and mixed emulsifiers was 1:4 and 1:5 and the oil concentration was 75-80%.

Regarding pH, the results revealed that VCO emulsion enriched with HA had pH values of 7.42 – 8.06.



**Figure 3:** Percentage of stability of VCO emulsion enriched with different concentration of HA solution. Different letters (a,b,c,d) above the columns indicate significant difference between eight groups under the same value HLB ( $P < 0.05$ )

### 3.4 The observation of w/o emulsion by microscopy

In w/o emulsion, the small droplets of water were penetrated in oil disperse phase. The observation by microscopy showed that, the droplets had blue color at the center which indicates that the emulsion is water in oil type. This result was supported by study of Flanagan (2006) [13], who found that the continuous blue phase surrounding the droplets indicate the emulsion is oil-in-water type and clear outer phase with dark bluish droplets indicate water-in-oil type of emulsion.

## 4 CONCLUSIONS

VCO could be performed into w/o emulsion by using the mixed emulsifiers of Span 80 and Tween 20 to reach the desired HLB value. The results showed that the proper HLB values to obtain the clear transparent w/o emulsion were 7, 8 and 9. The water phase of VCO emulsion could be substituted by hyaluronic acid solution up to 1 % w/v of concentration without interrupting the stability of emulsion. With HA supplementation, the VCO emulsion has additional ability to hydrate and moisturize the skin. From the results and discussions above, it can be concluded that the optimum formula for highest HA (1% w/v solution) enriched VCO emulsion is formula A, 1[HA:E] : 4[O], containing 3.32% w/w HA, 16.68 w/w E, and 80% VCO.

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## **EVALUATION OF HISTAMINE CONTENT IN TUNA FISH AND MACKEREL FISH BEFORE AND AFTER COOKING WITH PINEAPPLE AND GINGER BY HPLC-PDA METHOD**

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### **ABSTRACT**

A research of the variation of histamine levels in 30 tuna fish and 30 mackerel samples were studied by HPLC-PDA method. The results showed that concentration of histamine in fish samples increased after day 1st stored in cool of refrigerator and maximized at day 6<sup>th</sup> (11 ppm to 180 ppm). Histamine in fish samples before and after processing with pineapple, ginger had noticeable changes. The level of histamine in tuna samples decreased from 180.5 ppm to 20.6 ppm and 72.9 ppm when cooked with pineapple and ginger, respectively. Meanwhile, histamine levels in mackerel decreased from 163.4 ppm to 10.8 ppm and 50.8 ppm with ginger.

**Keywords:** Histamine, mackerel, scombroid, tuna fish

### **1 INTRODUCTION**

Biogenic amines are low molecular weight organic bases, they can be formed and degraded as a result of normal metabolic activity in animals, plants and microorganisms, in the latter case, biogenic amines may be used as an indicator of food spoilage [1]. These amines are usually produced in foods by decarboxylation of amino acids as the result of the decarboxylase. Histamine, derived from the decarboxylation of histidine (free amino acid that presents in the fish muscles) by decarboxylase enzyme that are released by some bacteria is a biological amine that affects local immune responses, regulation of intestine physiological functions and neuron transmitter in the enteric nervous system. Histamine is the causative agent of scombroid poisoning, a food-borne chemical hazard. Scombroid poisoning is usually a mild illness with a variety of symptoms, including rash, urticaria, nausea, vomiting, diarrhea, flushing, and tingling and itching of the skin [2]. Severity of the symptoms can vary considerably with the amount of histamine ingested and the individual's sensitivity to histamine [3]. Scombroid fish, such as tuna, mackerel, bonito, and saury, that contain high levels of free histidine in their muscle, are often implicated in scombroid poisoning incidents. However, several species of non-scombroid fish, such as mahi-mahi, bluefish, herring and sardine, have often been implicated in incidents of

scombroid poisoning [4]. Histamine fish poisoning (or scombroid poisoning) is of an important public health and safety concern [5]. The symptoms of histamine poisoning are nausea, vomiting, diarrhea, oral burning sensation, itching and rash [6]. Histamine has biologically strong effects such as directly stimulation of the heart causing extra vascular smooth muscle contraction or relaxation.

It stimulates both sensory and motor neurons and controls gastric secretion. Consequently, histamine fish Histamine in Canned Tuna Fish 835 poisoning have various signs or symptoms [7]. Symptoms are diminished by antihistamines or may be reduced by a histamine free diet [8,9]. In this research, the variation of histamine levels in 30 tuna fish and 30 mackerel samples were studied by HPLC-PDA method. The assessment of fish samples before and after processing with ginger, pineapple on characters created. Therefore, using pineapple to process fish may be reduced levels of histamine in fish.

## **2 MATERIALS AND METHODS**

### **2.1 Chemicals**

The chemicals were used for HPLC purified, Methanol (MeOH), Hexane,  $\text{KH}_2\text{PO}_4$ , Acetonitrile (ACN), 1-Heptanesulomic acid sodium salt, Standard histamine, Water deionized (DI)

- *Buffer solution, pH2.5*: Weighted 10.2g of  $\text{KH}_2\text{PO}_4$  and dissolved in 3 liters of water to swirl DI, adjusted with concentrated  $\text{H}_3\text{PO}_4$  acid to pH=2.5.

- *Standard solution of histamine 1000 ppm*: Accurately weighted 10mg histamine standard and transferred to 10ml flask and measured up to volume with methanol, the solution was stored in a refrigerator.

- *Mobile phase solvent*: The mixture of 25mM phosphate buffer (pH 2.5) –acetonitrile (9:1 ratio) was made by mixing 900ml of 25mM phosphate buffer (pH 2.5) with 100ml of acetonitrile (ACN) and 1.0g of 1-Heptanesulomic acid sodium salt (1g/1liter).

*Samples collected*: 30 fresh tuna and 30 mackerel fish samples were taken directly from Can Gio beach, Ho Chi Minh-City, Viet Nam. After washing, the fishes were put in boxes that contained ice (the ice layer is alternated with fish) and transported to the laboratory.

### **2.2 Analytical procedures:**

Weighted about 5 grams of sample, then put into 50ml centrifuge tube, shook for 15 minutes and then centrifuged until the sample was completely separated. Dissolved the residue in mobile phase solvent and removed methanol then added 2ml of hexane solvent to eliminate fat. The solution was transferred through the column C-18 to extract. After that, the solution was filtered by 45mm membrane filter and placed in vial container and it was injected into the HPLC-PDA system to analyze. The operating conditions of the equipment are summarized as follows: column

temperature: 45°C, wave length: 220nm, sample injection 1 mL/ min with auto injector, mobile phase composition KH<sub>2</sub>PO<sub>4</sub> buffer: ACN (9:1).

### 3 RESULTS AND DISCUSSION

#### 3.1 Calibration curve

After injecting histamine in following the standard concentration: 1ppm, 2ppm, 5ppm, 10ppm, 20ppm, 30ppm, 50ppm into the HPLC system with the parameters installed in order from low to high, we obtained chromatograms. Based on the standard chromatogram of histamine, we found that histamine retention time is 7 minutes. Therefore, histamine was detected after 7 minutes with analytical conditions and equipment above. From the results shown at the Figure 1, the relationship between the peak area in the obtained chromatogram with histamine levels were established. Calibration curve was constructed with a correlation coefficient of  $R^2=0.999$ , the standard curve should be accepted and used to calculate the next result.

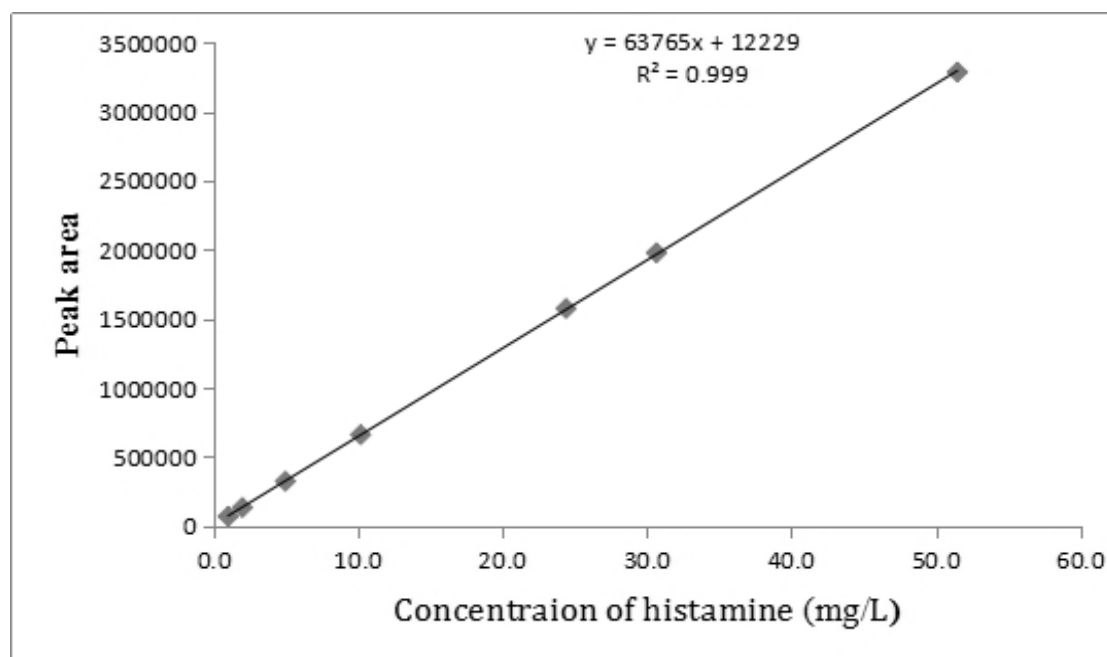


Figure 1: Calibration curve of histamine

#### 3.2 LOD and LOQ method

To determine the quantitative limits and detection limits, the spike in the standard histamine concentrations were prepared at: 10ppm, 20ppm, 30ppm, 50ppm in accordance with section 2.2 with the conditions of HPLC-PDA systems. The results after measuring and calculations were presented in Table 1.

**Table 1:** LOD, LOQ survey results

	Blank	Spike			
C, ppm	0	5 ppm	10 ppm	20 ppm	30 ppm
Area	159333	185644	640523	943427	1522642
S/N		1.2	4.0	5.9	9.6

From the results showed at Table 1, at the concentration of 10ppm with the ratio of  $S/N \approx 4$ , the limit of detection  $LOD=10\text{ppm}$  and quantification limits  $LOQ = 30\text{ppm}$  ( $LOQ = 3 \times LOD$ ) were found. Although, this method was not high sensitivities for the analysis but it was still used to analyze because of the high histamine levels in tuna and mackerel samples.

### 3.3 Recovery

**Table 2:** Results of the recovery process

Samples	m(g)	V(mL)	C spike, ppm	Area	Co,ppm	C sample (ppm)	R(%)	R (%)
1	5.135	10	50	1547884	24	47	94	97
2	5.066	10	51	1595698	25	49	96	
3	5.029	10	51	1578638	25	49	95	
4	5.086	10	51	1669669	26	51	101	
5	5.083	10	51	1624882	25	50	98	
6	5.147	10	50	1596504	25	48	96	

To examine the recovery of the method, six samples were prepared with the histamine concentration of 50ppm. The samples were kept in two days and analyzed by the HPLC system. The analytical results were recorded and calculated which were shown in Table 2. From this table, the recovery of all samples was all over 90%, so this method could be applied to analyze the levels of histamine in fish.

### 3.4 Changes in histamine levels by temperatures

The change in histamine concentration by temperature was studied by heating at 100°C, 150°C, 200°C for 30minutes of 50 ppm standard solution. After heating, the samples were filtered through 0.45µm filters and analyzed by HPLC system. The results were shown in Table 3.



**Table 3:** Histamine concentrations after heating at different temperatures

Temperatures	Before (C, ppm)	After (C, ppm)
25°C	50	41.9 ± 4.8
100°C	50	42.1 ± 4.2
150°C	50	42.8 ± 3.4
200°C	50	42.7 ± 3.8

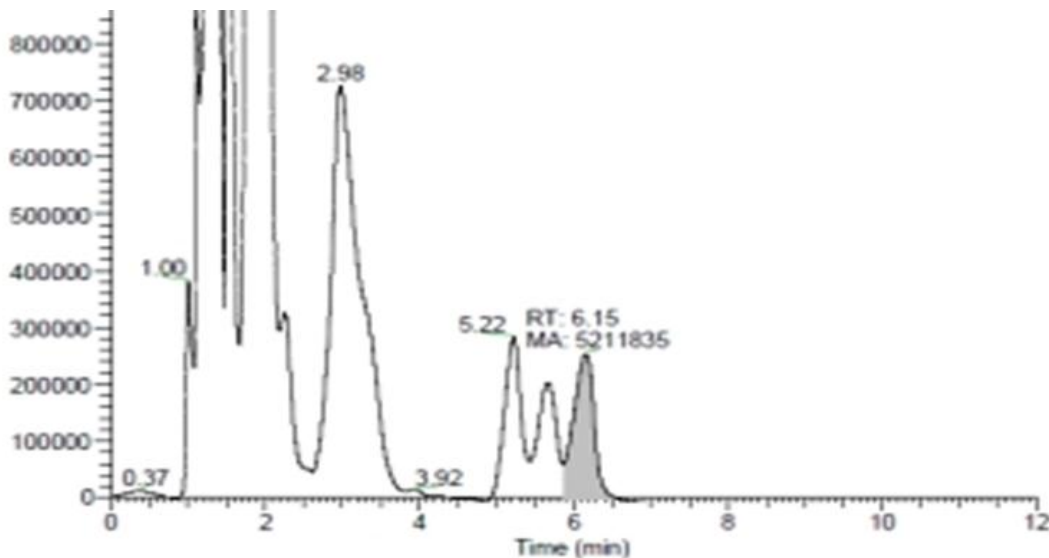
The results showed that despite being heated at different temperatures up to 200° C, the histamine level did not change significantly. It means that consumer still can be poisoned even though the fish containing histamine was cooked in high-temperature. The stability of histamine under high temperatures was also among the causes of mass poisoning when people used un-fresh fish.

### 3.5 Comparison of the histamin level change in storing in refrigerator cooling and frezeing zone

To evaluate the change of histamine level in tuna and mackerel during storage, we arranged two sets of samples from the same: a sample set was stored in the frezze zone of the refrigerator (4<sup>0</sup>c). Another one was stored in the refrigerator cooler zone (16<sup>0</sup>C). Samples were analyzed after each storage day. The analysis results were shown in the table 4.

**Table 4:** Conten of histamine in sample before and after store process

Days	Mackecrel		Tuna	
	Frezee store	Cool store	Frezee store	Cool store
1	ND <sup>*</sup>	0	11.0 ± 1.3	12.0 ± 1.3
2	ND <sup>*</sup>	0	12.0 ± 1.2	18.0 ± 1.2
3	ND <sup>*</sup>	11.2 ± 1.2	11.5 ± 1.4	26.5 ± 1.4
4	ND <sup>*</sup>	15.3 ± 7.4	12.0 ± 1.2	152.0 ± 16.2
5	ND <sup>*</sup>	163.4 ± 16.7	12.5 ± 1.1	180.5 ± 18.1
6	ND <sup>*</sup>	143.2 ± 14.2	13.2 ± 1.5	178.2 ± 21.5



**Figure 2:** Chromatography of histamine content after 2 day stored in refrigerator cooling zone (16°C)

The results showed that while histamine level was not significantly change storing in the freezing zone of the refrigerator. But it rapidly increased keeping in the cooling compartment on the 3th day and reached to the highest level on the 5th day of storage. This could be explained as when the fish was rotten, microorganisms such as *Enterobacteriaceae*, *Morganella morganii*... grew and generated enzymes to convert the amino acid histidine to histamine and thereby increasing histamine levels. However, in low temperature environment, these bacteria may almost be inactive so it was not able to convert amino acid histidin to histamine. Therefore, freezing fish is one among the ways to limit the development of histamine levels of during storage.

### 3.6 Researching the change of histamine levels before and after processing with ginger and pineapple

To study the change in histamine content during processing with ginger and pineapple. Histamine-infected fish samples after 5 days of storage in the cooler were selected for testing. These samples were boiled for 30 minutes. The remaining samples were washes for analysis, the results of the analysis were presented in the Table 5.

**Table 5:** Content of histamine in sample before and after cooking

Samples	Before	After washed	After processed with Ginger	After processed with Pineapple
Spike	100	98.4 ± 10.5	40.5 ± 5.5	12.4 ± 1.6
Tuna	180.5 ± 18.1	170.3 ± 13.6	72.9 ± 6.7	20.6 ± 2.3
Mackerel	163.4 ± 16.7	168 ± 11.8	50.8 ± 6.8	10.8 ± 1.1

After processing with pineapple, the histamine levels in Tuna dropped from 180.3 ppm to 20.6 ppm. Meanwhile, histamine level in Mackecrel decreased from 163.4 ppm to 10.8 ppm. The

changes in the concentration of histamine in the analysis results were remarkable. The reason could be that the pineapple contains a certain amount of acid, which reacted with histamine and converted to salt. So, pineapple can be used to cook with tuna and mackerel to eliminate a part of histamine. This also explains why in the old days, people often cooked tuna, mackerel with pineapple, as it could both create delicious taste, and reduce the toxicity of histamine in these fish.

#### **4 CONCLUSIONS**

Based on the results of the experiment that conducted to determine the levels of histamine in tuna using mackerel HPLC system with PDA detector, we obtained the following results:

- Histamine concentration was not affected by temperature. Therefore, when fish contained histamine toxicity even at high temperature processing, the histamine poisoning remained to users.
- Research results showed that fish samples before and after processing with pineapple, ginger had noticeable changes. The level of histamine in tuna samples decreased from 180.5 ppm to 20.6 ppm and 72.9 ppm when cooked with pineapple and ginger, respectively. Meanwhile, histamine levels in mackerel decreased from 163.4 ppm to 10.8 ppm and 50.8 ppm with ginger.

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## **BIOACCUMULATION, DEPURATION OF HEAVY METALS (As, Cd, Pb) AND METABOLISM OF THESE METALS IN BODY OF MUSSELS (*MARETRIX LYRATA*) DURING 20 DAYS IN ARTIFICIAL MEDIA OF CULTURE**

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### **ABSTRACT**

The purpose of this work is to estimate the extent of As, Pb, Cd bioaccumulation by *Meretrix Lyrata* mussels in artificial media of culture contaminated by heavy metals (As at 1.5 and 2.5ppm, Pb at 1.5 and 2.5ppm, Cd at 0.1, 0.5, 1ppm) during the first five days and release of these heavy metals in clean water during the following 15 days. Quantitation was performed by ICP and AAS with Hydride System. Bioaccumulation increased when heavy metal concentrations in water increased and in the order Pb>Cd>As. Release was also observed to increase with the increasing amounts of absorbed metals. After 20 days of experiments, following figures of metal release were obtained: As ~100%, Pb ~ 68.9%, and Cd~39.65%. In the case of Cd contamination, no mussel survived after 10 days of experiments even at low Cd concentration of 0.1ppm.

Residue of heavy metals in the body of mussels was metabolized to another chemical form. Inorganic Cd was metabolized to Cd-metallothionein and detected by LC/MS ESI (+). As was metabolized to monomethyl arsenic acid (MMA), dimethylarsenic acid (DMA) and detected by HPLC-UV- AAS- HG, and Pb was metabolized to phosphate hydroxyl lead (Pb<sub>5</sub>(OH)(PO<sub>4</sub>)<sub>3</sub>) - which was detected by XRD.

**Keywords:** As, Cd, Pb, MMA, DMA, Cd7- MT, phosphate lead.

### **1 INTRODUCTION**

Mussels (*Meretrix lyrata*) live at warm sea of Western Pacific Ocean from Taiwan to South of Vietnam. Mussels have the ability to accumulate heavy metals and contaminated organisms in culture medium [5,7]. The bivalves are very popular for consume and export because they have some advantages such as high protein, minerals, low cholesterol and lipid as compared to crab,

shrimp [6]. Besides nutrition value, bivalves are proposed as bio-indicator for water and sediment environment due to high ability of bioaccumulation and sensitivity to the changes in the environment [1].

Food safety is a very important issue in Vietnam and many countries in the world. There is a risk arising from consumption of polluted mussels [3]. For this reason, this study was aimed to estimate the extent of As, Pb, Cd bioaccumulation by *Meretrix Lyrata* mussels in artificial media of culture contaminated by heavy metals, and release of these heavy metals in clean water. In addition, the metabolism of heavy metals when they are accumulated in body of mussels were also studied.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Mussels (*Meretrix lyrata*) and sea water were collected at Can Thanh beach of Can Gio province, Ho Chi Minh City and then brought to Thu Duc experimental farm. Mussels were cultured and exposed to heavy metals at different concentration (As, Pb at 1.5 and 2.5 ppm, Cd at 0.1, 0.5, 1.0 ppm) for 5 days for accumulation. Then contaminated water was replaced by clean sea water for next 15 days and release of heavy metals from the mussels was monitored.

### 2.2 Model of artificial culture of mussels

Each tank was filled with sea water contaminated with heavy metals. All parameters such as pH, NO<sub>3</sub>, NH<sub>4</sub>, number of dead mussels were monitored every day. Mussels samples were collected and brought to laboratory for analysis

**Table 1:** Model of artificial culture of mussels for each experiment (□ tank experiment)

	Control mussel	As contaminated	Cd contaminated	Pb contaminated
3days	□ □ □ □	□ □ □ □	□ □ □ □	□ □ □ □
5days	□ □ □ □	□ □ □ □	□ □ □ □	□ □ □ □
10days	□ □ □ □	□ □ □ □	□ □ □ □	□ □ □ □
15days	□ □ □ □	□ □ □ □	□ □ □ □	□ □ □ □
20days	□ □ □ □	□ □ □ □	□ □ □ □	□ □ □ □

## 2.3 Equipment used for analysis

Analysis of heavy metals: Cd, Pb were analyzed by using ICP Perkin Elmer 5300, and As was analyzed by AAS – Hydride System.

Analysis of metabolite of heavy metal: Cd - Metallothionein was analyzed by LC/MS ESI (+), MMA, DMA was analyzed by AAS-UV-HG, and  $(\text{Pb}_5(\text{OH})(\text{PO}_4)_3)$  was analyzed by XRD.

## 3 RESULTS AND DISCUSSION

### 3.1 Accumulation of trace metals: Cd, Pb, As in in-complex form: $\text{CdCl}_2$ , $\text{Pb}(\text{CH}_3\text{COOH})_2$ , $\text{As}_2\text{O}_3$

Mussels (*Meretrix lyrata*) were cultured in contaminated water for 3 and 5 days. The results obtained in the table 2 show that the concentration of heavy metals strongly affected the ability of uptake and accumulation of heavy metals in mussels. High concentration of metals led to high accumulation of metals in mussels. All of three metals which accumulated in mussels were increased from first day to fifth day. Particularly for lead, content of Pb in mussels was 5.4ppm after 5 days exposed to concentration of 2.5 ppm Pb, lower than that in mussels (9.97 ppm) when exposed to concentration of 1.5 ppm Pb in artificial medium. It can be explained that, when uptake of Pb reaches maximum, mussels will depurate [7], therefore the process of Pb accumulation by mussels probably reached its peak after 3<sup>rd</sup> day and before 5<sup>th</sup> day.

**Table 2:** Accumulation of As, Cd, Pb in tissue of mussels exposed to different dissolved concentrations for 3 days and 5 days in artificial medium

Heavy metal	Control mussels (mg/Kg)	3 days ( mg/Kg)	5 days (mg/Kg)	In comparison with control mussels (times)
As - 1.5mg/L	1.01	1.55	1.53	1.5 times
As - 2.5mg/L		1.56	1.79	1.7
Pb - 1.5mg/L		4.10	<b>9.97</b>	207.7
Pb - 2.5mg/L	0.045	4.97	5.40	135
Cd - 0.1mg/L		0.74	0.80	4.0
Cd - 0.5mg/L	0.2	2.50	<b>3.20</b>	16.0
Cd - 1.0mg/L		2.88	<b>3.48</b>	17.4
Cd – 1.5mg/L		dead	dead	ND

The uptake characteristic of each heavy metal by mussels was different. As and Pb concentration in artificial medium was the same (1,5ppm and 2,5ppm) but accumulation was different: at 5<sup>th</sup> day, content of As and Pb in mussels reached value 1.79 ppm and 9.97ppm respectively. Cd is highly toxic for mussels. When mussels were exposed to 1.5 ppm of Cd in the medium, all

mussels were dead after one day. At small concentration of Cd in the medium (0.1 ppm, 0.5 ppm, 1 ppm), mussels could survive until 10<sup>th</sup> day of experiment and after that they all also were dead. Cd content in mussels were 3.48 ppm and 3.2 ppm on the 5<sup>th</sup> day when mussels were exposed to 1ppm and 0.5 ppm Cd in the medium, respectively. At Cd 0.1ppm in the medium, content of Cd in mussel was very small (0.80 ppm). Thus, bioaccumulation of heavy metal by mussels at 5<sup>th</sup> day increased in the order: Pb (9,97ppm) > Cd (3.48, 3.2ppm) > As (1.79 ppm).

### 3.2 Depuration of trace metals Cd, Pb, As in mussels

**Table 3:** Residue of trace metals: Cd, Pb, As in mussel (*Meretrix lyrata*)

Metal in water	Control mussel (mg/kg)	Depuration period				Average Depuration
		5 <sup>th</sup> day (mg/kg)	10 <sup>th</sup> day (mg/kg)	15 <sup>th</sup> day (mg/kg)	20 <sup>th</sup> day (mg/kg)	
As - 1.5mg/L	1.0	1.53	1.05	1.02	1.02	> 90%
As - 2.5mg/L		1.79	1.42	1.02	1.02	
Pb - 1.5mg/L	0.045	<b>9.97</b>	4.90	3.20	3.20	<b>68.9%</b>
Pb - 2.5mg/L		5.40	3.90	3.30	3.20	
Cd - 0.1mg/L	0.2	0.80	0.83	Mussels were dead		<b>40%</b>
Cd - 0.5mg/L		<b>3.20</b>	1.80			
Cd - 1.0mg/L		<b>3.48</b>	2.10			

After five days, contaminated water was changed to clean water for study of depuration. As compared to 5<sup>th</sup> day and the control mussels, after 10 days, content of heavy metals in body of mussels decreased, although not by same degree.

Results in table 3 show that Arsenic (As) content in mussels continued to decrease during the remaining days. From day 15<sup>th</sup>, mussels excreted more than 90% of As accumulated. An equilibrium between As bioaccumulation and excretion would be reached then. Mussels continued to live healthy after 20 days. For lead (Pb), after five days, Pb content in mussels continued to decrease up to day 20<sup>th</sup> at both concentrations of Pb in the medium. However, mussels did not excrete all of bio-accumulated Pb. In comparison with the control mussels, the amounts of retained Pb were substantial. Mussels eliminated about 69% of accumulated lead and continued to live healthy after 20 days.

For cadmium (Cd), after 5 days the Cd content in mussels decreased. Mussels eliminated about 40% of Cd from body and remaining content of Cd in body of mussels was about 60%. In comparison with the control mussels, the amounts of retained Cd increased in the following order of Cd concentration in the medium: 1 ppm Cd > 0.5 ppm Cd > 0.1 ppm Cd. At 0.1ppm Cd, no



elimination of Cd by mussels was detected. Cd is highly toxic to mussels so all mussels were dead after 10 days of experiment even at the lowest Cd concentration of 0,1ppm. Control mussels continued to live healthy after 20 days of experiment.

### 3.3 Metabolism of remaining heavy metal in mussels

When mussels were exposed to metals in contaminated artificial medium, mussels can uptake metals and accumulate these metals in their body. They can also depurate metals from body. However, mussel cannot eliminate all metals which they accumulated. Thus, the question is in what form do the metal ions remain in the mussel's body?

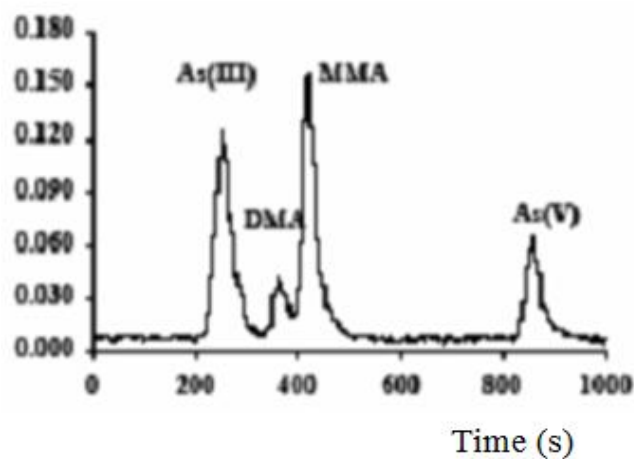
#### 3.3.1 Arsenic metabolism

In many species arsenic metabolism is characterized by two main reactions: First, reduction of pentavalent to trivalent arsenic  $\text{As(V)} \rightarrow \text{As(III)}$ . Next oxidative methylation reaction in which trivalent forms of arsenic are methylated to form monomethylarsenic acid  $[(\text{CH}_3)\text{AsO}(\text{OH})_2]$ , dimethylarsenic acid  $[(\text{CH}_3)_2\text{AsO}(\text{OH})]$  and trimethylarsenic acid  $[(\text{CH}_3)_3\text{AsO}]$ . Methylated arsenic forms are rapidly excreted from the body of mussels [1, 2]. Mussel samples after 3 days and 20 days were used for identification of MMA, DMA by HPLC–AAS-UV-HG equipment. Results obtained are presented in the table 4 and figures 1-4.

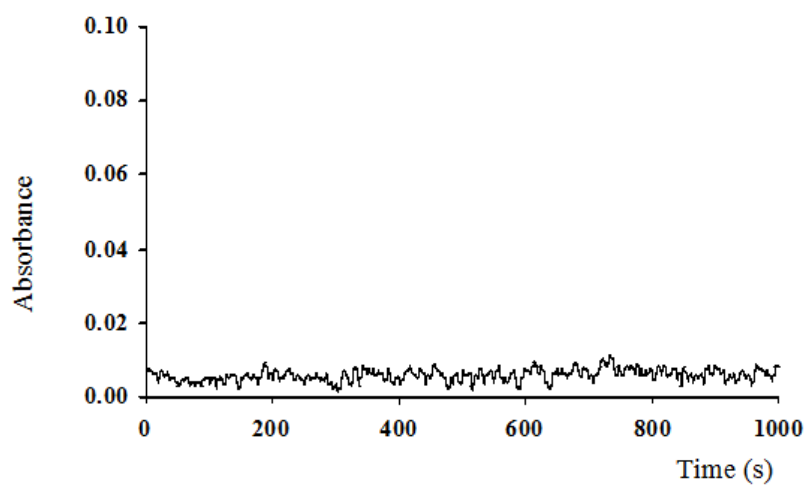
**Table 4:** Metabolism of As in the mussels for 3 days and 20 days in

N <sup>o</sup>	Sample	As(III) (ppb)	DMA (ppb)	MMA (ppb)	Arsenobetain (ppb)	As(V) (ppb)
1	Standard solution	25	25	25	25	25
2	control mussel	ND	ND	ND	ND	ND
3	Contaminated mussel in 3 days	357	ND	ND	ND	32.5
4	Contaminated mussel in 20 days	8.7	7.2	6.1	ND	15.6

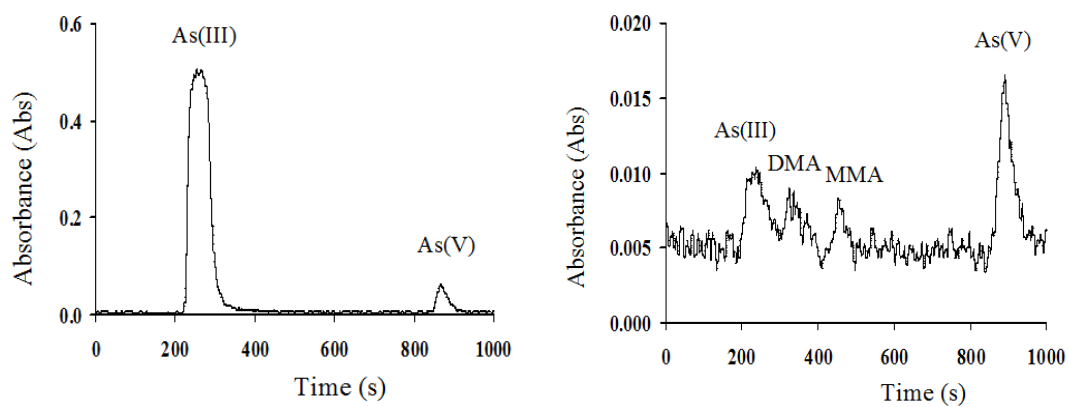
ND: not detected



**Fig. 1:** Standard chromatography As(III): 25 µg/L; DMA: 25 µg/L; MMA: 25 µg/L; As(V): 25 µg/L



**Fig. 2:** Control



**Fig. 3:** Contaminated mussel in 3 days

Mussels exposed to As for 3 days and 20 days were treated by methanol solution and the extracts were used for identification of the arsenic forms: As(III), As(V), MMA, DMA and arsenobetain. Results presented in table 3 show that control mussels did not content any arsenic form. Mussels exposed to As for 3 days contained only As (III) and As(V). All arsenic forms appeared in mussels exposed to As for 20 days. In conclusion, there were the methylated forms of As in mussel body, so mussels can detoxify and depurate toxic substances to the environment.

### 3.3.2 Metabolism of Cd

Cadmium (Cd) can bind to special small protein molecule (6000DA – 10000DA) to become MT-metallothionein and complex of MT with metal (Me –MT) [4, 6]. So Cd accumulated in mussel body will be converted to Cd<sub>7</sub> – MT. In order to identify Cd metabolism in mussel, LC/MS ion trap was used. By SIM of LC/MS method, peaks of MT-1 and MT-2 were found on Rabbit liver MT-1 standard. Then extracts of mussels exposed to Cd for 10 days were analyzed by LC/MS and all peaks of MT-1 and MT-2 were found. Results are presented in table 5 and figure 5.

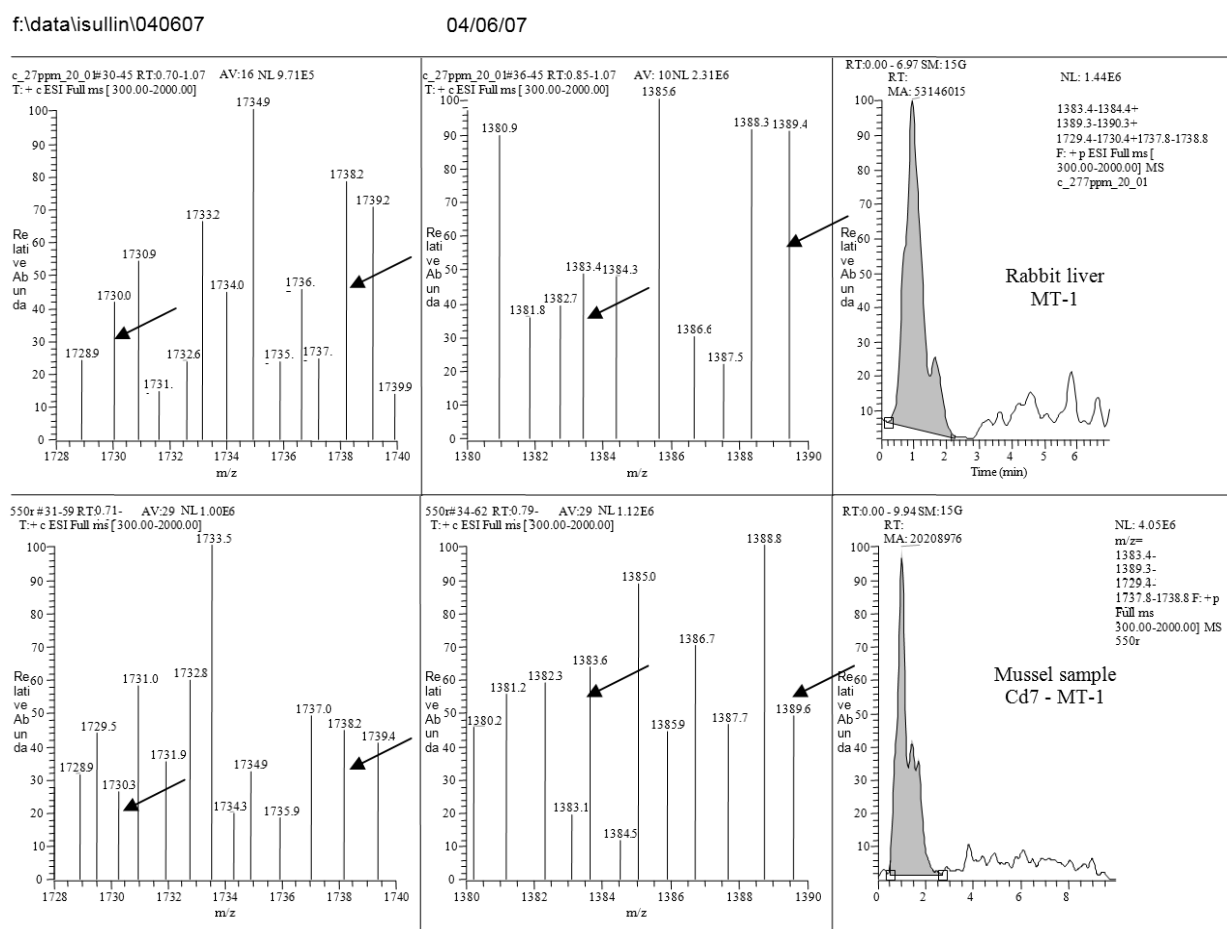


Fig. 4: Chromatography of Standard MT-1 and mussel sample

**Table 5:** Results measured of MT-1 of standard and mussel sample by LC/MS ion trap)

Cd <sub>7</sub> MT-1 Rabbit liver				Cd <sub>7</sub> MT-1 – mussel samples	
MT-1	M <sub>w</sub> (Cd <sub>7</sub> MT-1)	M <sub>w</sub> [5H <sup>+</sup> ]	M <sub>w</sub> [4H <sup>+</sup> ]	Mw[(5H <sup>+</sup> )]	Mw[4H <sup>+</sup> )]
α (a)	6917	1384.4	1730.2	1384.1	1730.1
β	6949	1390.8	1738.2	1390.7	1738.0
γ	6965	1394.0	1742.2	1392.7	1741.9
δ	6988	1398.6	1748.0	1399.1	1747.7
ε	7013	1403.5	1754.2	1402.9	1753.7

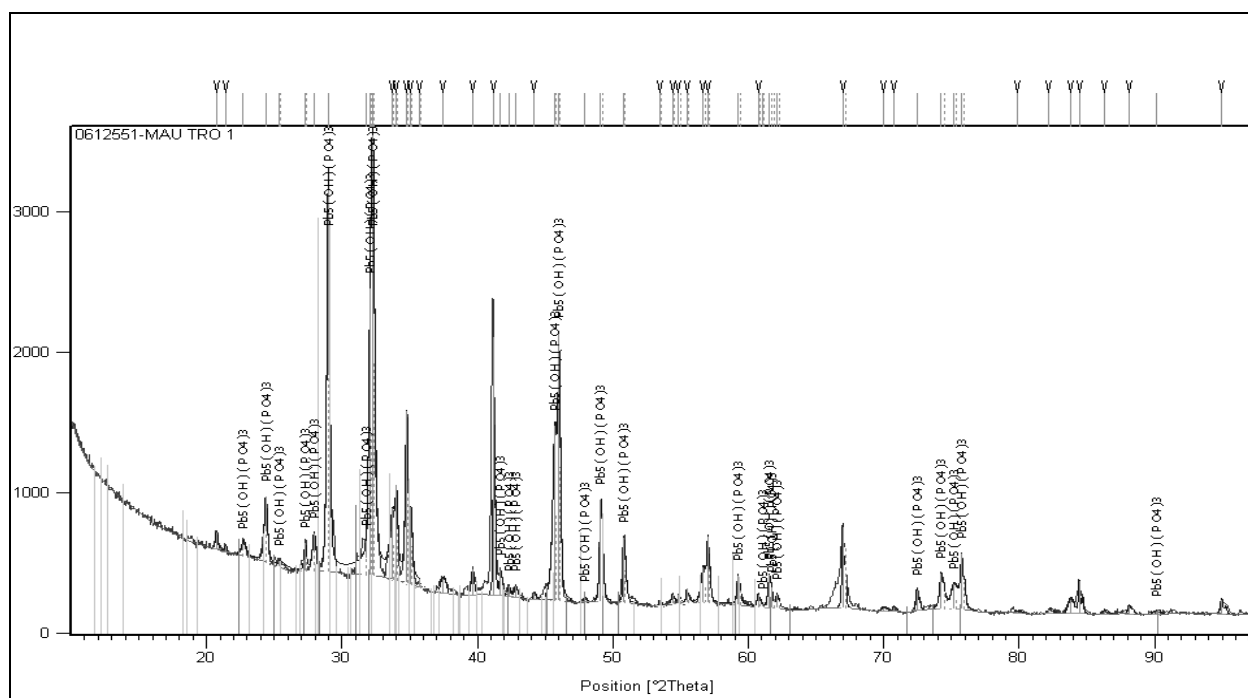
Similar to arsenic, mussels metabolized Cd to metallothionein and complex Cd<sub>7</sub>-MT and detoxify by themselves. However, complex Cd-MT was not easily depurated from mussel body and it was accumulated in mussels for a long time [4, 6]. Structure of Cd<sub>7</sub> – MT of mussel (*Meretrix lyrata*) was similar to structure of Cd<sub>7</sub> – MT of Rabbit.

### 3.3.3. Metabolism of Pb

Remaining content of Pb in mussel body was 32%. It is known that when Pb accumulates in organism, it may be transported to lead phosphate and stored in kidney [3, 5]. Mussels of 20 days exposed to Pb were analyzed. Mussel samples were burned at 500<sup>0</sup>C and ash was obtained to identify the chemical forms of Pb by XRD equipment. Results are presented in the table 6 and figure 6.

**Table 6:** Chemical compounds of Pb in mussels sample in ash by XRD equipment

Chemical compounds	Chemical forms
Lead phosphate hydroxy	Pb <sub>5</sub> (OH)(PO <sub>4</sub> ) <sub>3</sub>
Potassium chloride and sodium chloride	KCl, NaCl
Mixed metal phosphate	Na <sub>2</sub> CaMg(PO <sub>4</sub> ) <sub>2</sub>



**Fig. 5:** XRD chromatography of Pb metabolites in mussels

## 4 CONCLUSIONS

Accumulation of heavy metals in mussel (*Mytilus edulis*) depends on chemical properties and concentration of metals. Uptake of metals in mussels increased with increasing concentration of metals in the culture medium but not in proportion ( $Pb > Cd > As$ ).

Depuration of metals depends on chemical properties of metals, but not on the concentration of metals in the culture medium. As was depurated about 90%, Cd 40% and Pb about 69%.

Modern analytical equipment were successfully applied for identification of metabolites of the remaining metals in mussels (As by HPLC–UV–AAS–Hg, Cd by LC/MS–SIM and Pb by XRD).

Metabolisms of heavy metals:

As (V) → reduced form As(III) → methylated forms MMA, DMA → fast depuration

Cd → metallothionein → Cd<sub>7</sub> – MT → slowly depuration

Pb → Pb<sub>5</sub>(OH)(PO<sub>4</sub>)<sub>3</sub> → storage in kidney → slowly depuration

Mussels have ability to accumulate and retain toxic metals As and Pb at high levels. Mussels should be carefully treated for the toxic metals before consumption.

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## DETECTION AND QUANTIFICATION OF AURAMINE O CONTAMINATION LEVEL IN MARKET FOODS

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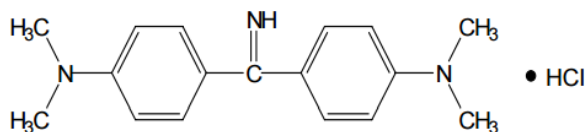
### ABSTRACT

This study was aimed to examine the contamination level of Auramine O (AO) in local market foods. The work contained both detection and quantification of AO in suspected foods sold in street markets and supermarkets in Ho Chi Minh City. Thin layer chromatography (TLC) was applied for quick detection of AO based on its fluorescence characteristic under UV light. To quantify the level of AO in foods, ultra-violet visible spectrophotometer at 339 nm of wavelength was used. Among 120 food samples collected randomly, there was 25±5.6% of total samples contaminated with AO, all of which belonged to a category of small-scale food products purchased from local or street markets. None of food samples from supermarkets showed the presence of AO. Among the positive samples, solid foods took the largest portion which was 15.3% while powder samples made 8.3% among total food samples. AO is an industrial dye banned in food all over the world, its level of contamination in this study, however, ranged from 3 to 19 ppm, which was hundred times higher than the data reported recently from the investigation in the local market foods.

**Keywords:** Auramine O, contamination, TLC, UV-vis

### 1 INTRODUCTION

Auramine O (AO) is a diarylmethane dye which is soluble in water (10mg/ml), ethanol (20mg/ml) and ethylene glycol monomethyl ether (60mg/ml). It is presented in the form of yellow needle crystal or yellow-to-brown powder [1]. Its molecular weight is 303.83g/mol and its melting point is from 250°C or higher. The molecular formula of AO is C<sub>17</sub>H<sub>21</sub>N<sub>3</sub>.HCl which chemical structure is showed in Fig. 1.



**Figure 1:** Chemical structure of Auramine O

AO colorant is used for dyeing of leather, jute, tanned cotton, and paints, and as dye components in inking ribbons, ballpoint pastes, oils and waxes, and carbon paper (IARC, 2010). AO is used as fluorescent staining agent to stain acid-fast bacteria in microbial analysis [2].

Several cases have been reported about the consumption or exposure to AO for certain time which increases the incidence of bladder tumours in human (Müller, 1933; Case et al., 1954). It is also reported about the damage of DNA induced by AO in liver, kidney and bone marrow of rats and mice, and in a human cell line [3].

Because of the potential harmfulness of AO, its uses in foods and cattle foods has been banned in many countries for years [4, 5]. Nevertheless, it was reported to be present in certain kinds of food such as bean products in China [6], pickled tea leaves in Singapore, Brunei [7], and cinnamon quills in New Zealand [8].

The wide-range investigations on the dyed-bamboo shoots and other food products in Vietnam, particularly in Da Nang City, Ho Chi Minh City and some North-middle provinces reported that 7 out of 9 samples of bamboo shoot in Da Nang was positive with AO [9]. Also, AO was found in 4 out of 4 samples of boiled and dried bamboo shoots in a local market in Ho Chi Minh City. The 2 samples of dried bamboo shoots contained AO with the amount about 11.84 µg/kg and 41.35 µg/kg, much higher than values in the boiled bamboo shoots (17.06 µg/kg and 3,108.94 µg/kg) [10]. AO was also found in pickled cabbages, chicken skin and Quảng noodles [11, 12, 13]. These are common daily foods widely used in Vietnamese food cuisine. Long-term consumption of them would probably result in certain diseases and influence human health.

There was a great deal of researches about AO contamination in foods. Tatebe et al. [14] developed a rapid, simple and low-cost thin layer chromatography method for the detection of AO by observing the yellow fluorescent spots at 254 nm when using more than 5 µL of sample solutions. On the other hand, AO was detected by measuring and comparing the fluorescent intensities of the solutions with those of the standard solutions containing bovine serum albumin [15].

High performance liquid chromatography (HPLC) has been widely used to quantify the amount of AO in food because of its high sensitivity and high accuracy [14]. However, high technical skills and high trained personnel are required.

Spectrophotometry is a simple and low-cost quantification method for either azo dyes or other chemicals. It was used to detect the amount of both permitted and non-permitted dyes in food in India [16]. Jingjing et al. [15] implied that it is possible to apply the use of bovine serum albumin to quantify AO level in food by using UV-Visible spectrophotometer.

This research was done for quick screening of AO in a wide variety of market and supermarket foods by thin layer chromatography method and the quantification of AO level in those positive food samples by UV-vis spectrophotometry. In conclusion, it was aimed to evaluate the



prevalence of AO-contaminated food and to give recommendation for consumers when choosing foods.

## **2 MATERIALS AND METHODS**

### **2.1 Collection of food samples**

There were 120 food samples divided into 2 groups: small scale and industrial scale products. They were divided into 2 states: solid (S) and powder (P).

The solid food samples were boiled bamboo shoots (BB), dried bamboo shoots (DB), pickled cabbages (PC), chicken skin (CS), dried tea leaves (DTL) and dried jackfruit (DJ). On the other hand, the powder food samples were 5-seasoning powder (FS), turmeric powder (TP), curry powder (CP) and Hue-noodle seasoning powder (HN).

Small-scale food samples were purchased from six different local markets or street vendors in six districts in Ho Chi Minh City and coded. Industrial-scale food samples were purchased from local supermarkets in Ho Chi Minh City and coded by following the branded name or the district of location. All food samples were kept at proper temperature.

### **2.2 Chemicals**

Auramine O standard powder and bovine serum albumin (BSA) were from Merck (Germany). Absolute ethanol, hexane, methanol, hydrochloric acid and 2-butanone were from Prolabo. Ethyl acetate, ammonium formate, sodium hydroxide, sodium chloride, acetic acid, boric acid and phosphoric acid (AR grade) were purchased from local suppliers.

### **2.3 Thin layer chromatography (TLC) detection method**

Saturated NaCl containing 0.1M NaOH was prepared by dissolving 04 g NaOH in 1L of the NaCl solution. A 1.6M HCOONH<sub>4</sub> (pH 2.5) was prepared by dissolving 10g of HCOONH<sub>4</sub> in 50mL of water and adjusted to pH 2.6 with formic acid. The solvent was a mixture of 2-butanone-methanol-1.6M ammonium formate (pH 2.5) (7:2:7 - v/v/v) [14].

Standard AO solution was prepared by dissolving AO standard powder in water to obtain 10 ppm solution concentration. It was then diluted to 12 different concentrations (1, 0.5, 0.1, 0.09, 0.08, 0.07, 0.06, 0.05, 0.04, 0.03, 0.02 and 0.004 ppm).

All solid samples were finely ground. 05 g of sample was dissolved in 20 mL of a solution containing 0.1M HCl:Ethanol (1:2 v/v) then it was shaken for 01 min. 20 mL of ethyl acetate was added into the mixture and it was shaken for 01 min again. After that, the solution was centrifuged at 3000 rpm for 2 min to get the supernatant. The procedure was repeated 2 times for the residues and the supernatants were combined in another Erlen flask.

01 mL of 2.5M NaOH and 50 mL of saturated NaCl (containing 0.1M NaOH) were added into the mixture, shaken for 01 min then the latter layer was removed. Next, 40 mL of hexane and 20

mL of 0.1M HCl were added and well-homogenised. The basic colorant was collected from the upper layer and kept in a 100 mL beaker.

07 points were marked on the start line of the silica gel plate. 05  $\mu$ L of 10 ppm of standard AO solution and 06 different food sample solutions were spotted on the marked points. The silica gel plate was then put into a TLC chamber containing around 100 mL of the solvent mixture. When the solvent reached the finish line, the plate was removed and dried at room temperature prior to being observed under white light and UV light.

Any yellow spots were marked and measured the moving distance then compared to the standard spot.  $R_f$  was then calculated by taking the ratio between the distance moved by spot and the distance moved by solvent.

## **2.4 UV-Visible spectrophotometry quantification method**

Britton-Robinson buffer solution contained 0.04M of  $\text{CH}_3\text{COOH}$ ,  $\text{H}_3\text{PO}_4$  and  $\text{H}_3\text{BO}_3$  (adjusted to pH 7.0 by 0.2M NaOH). Standard 0.0001M bovine serum albumin (BSA) solution was prepared by dissolving BSA powder in distilled water [15].

05 g of sample was mixed with 30 mL of ethanol 70% prior to being treated with ultrasound for 10 min. After that, it was centrifuged for 15 min. The solution was separated into another flask before adding 01 mL of BSA and 01 mL of BR buffer solutions. Wait for 10 min then it was subjected for UV-vis spectrophotometric analysis.

Standard samples were prepared by mixing finely-cut homemade pickled cabbage with standard AO solutions with different concentrations. Distilled water was used as blank for spectrophotometric measurement at  $\lambda=339\text{nm}$ .

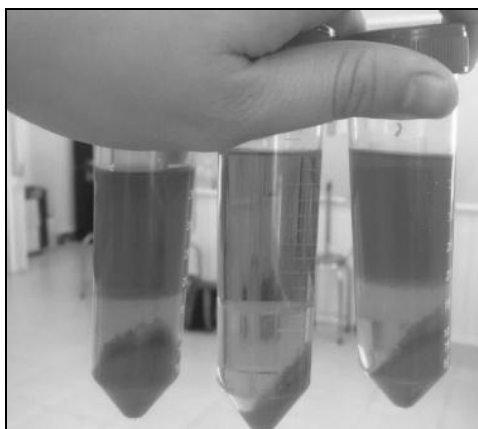
## **2.5 Statistical analysis**

Independent t-test (at  $p=0.005$ ) was used to analyse the collected data to prove the reliability of the presence of AO in Vietnamese foods, using standard statistical software SPSS ver. n 23.0.

# **3 RESULTS AND DISCUSSION**

## **3.1 Thin layer chromatography detection method**

After adding ethyl acetate, only the mixture of food samples containing AO was separated into 02 completely different layers: one had yellow or red transparent colour and one had opaque yellow or red colour, which depended on the original colour of the food samples (Fig. 2, 3).



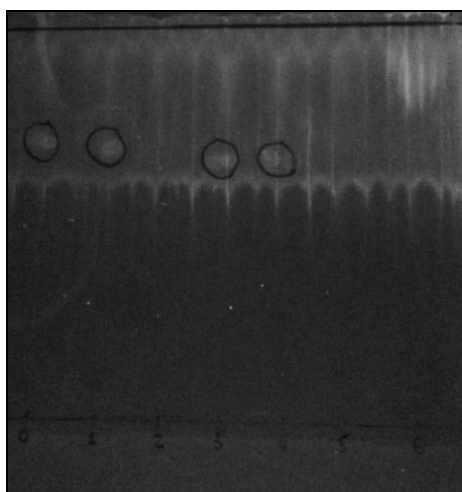
**Figure 2:** Mixture of boiled bamboo shoots after adding ethyl acetate, prior to centrifugation



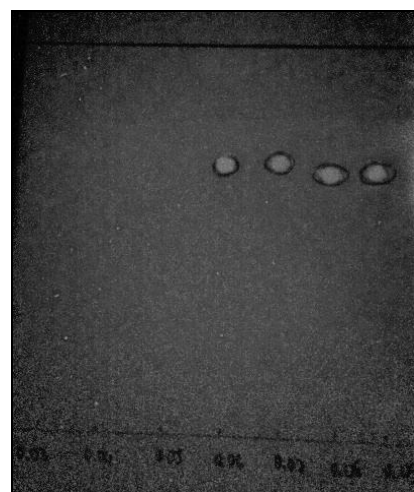
**Figure 3:** Mixture of turmeric powder after centrifugation

In both Fig. 2, 3, it could be clearly seen that there were two distinguishable layers of assorted colours and transparency. Those having a transparent upper level were the positive samples based on the TLC's results.

After conducting validation TLC using standard samples mixed with varied concentration AO solutions, the fluorescent yellow spots were found when the AO concentrations were 0.06 ppm or higher. The immersing time was around 1 h and 20 min for the solvent and spots to move the marked positions (Fig. 4, 5).



**Figure 4:** Validation TLC result, yellow fluorescent spot found when concentration was 0.06 or higher



**Figure 5:** Yellow fluorescent spots represented for positive turmeric samples

$R_f$  was measured and statistical calculations were conducted, which was reported in the table below. The average distance moved by spots was  $0.64 \pm 0.0186$  cm. The test was highly precise due to the 2.91 CV% (Table 1).

**Table 1:** Validation TLC results

Concentration (ppm)	Distance moved by solvent (cm)	Distance moved by spots (cm)	R <sub>f</sub>
<b>1</b>	10.5	7.1	0.6762
<b>0.5</b>	10.5	6.8	0.6476
<b>0.1</b>	10.5	6.7	0.6381
<b>0.09</b>	10.5	6.6	0.6286
<b>0.08</b>	10.5	6.6	0.6286
<b>0.07</b>	10.5	6.7	0.6381
<b>0.06</b>	10.5	6.5	0.6190
<b>Mean</b>			<b>0.6395</b>
<b>Standard deviation</b>			<b>0.0186</b>
<b>CV%</b>			<b>2.91%</b>

The silica gel plates were observed under white light and UV light. It was difficult to recognise the yellow spots under white light because of its yellow colour. On the other hand, it was easy to observe the fluorescent yellow spots under the UV light.

There were 15 out of 120 food samples concluded to contained AO ( $12.5 \pm 3\%$ ). None of them belonged to either solid or powder food products purchased from the supermarkets. All 15 positive results were associated with small-scale food products, which were mostly in solid form (total 11 samples of boiled bamboo shoots, dried bamboo shoots and pickled cabbages). Only 04 samples of turmeric powder and curry powder were concluded to contain AO (Table 2).

**Table 2:** Ratio of positive samples detected by TLC

Categories	Total samples	Total positive samples	$\hat{p}$	$\hat{q}$	SE	95% CI		Positive percentage (%)
						Min	Max	
<b>Small scale</b>	60	15	0.250	0.750	0.056	14.04%	35.96%	25
<b>Industrial scale</b>	60	0	0	1	0	0	0	0
<b>Solid</b>	72	11	0.153	0.847	0.042	6.97%	23.59%	15.3
<b>Powder</b>	48	4	0.083	0.917	0.040	0.51%	16.15%	8.3
<b>Total</b>	120	15	0.125	0.875	0.030	6.58%	18.42%	12.5

According to the statistical analysis results, there was no industrial scale food samples contaminated with AO. Meanwhile, there was  $25 \pm 5.6\%$  of small-scale food samples containing AO.

In terms of solid samples, there was  $15.3 \pm 4.2\%$  of random solid food samples positive to AO while the number for random powder food samples was  $8.3 \pm 4.0\%$ .

Based on the results of independent samples t-test using SPSS software, there was a significant difference in the AO contamination between small scale samples and industrial scale samples ( $t_{118} = 4.435$ ,  $p=0$ ).

However, there was no significant difference in the AO contamination between solid and powder samples ( $t_{118} = 1.123$ ,  $p=0.264$ ).

### 3.2 UV-Visible spectrophotometry quantification method

According to the calibration equation  $y=0.0184x$ , it can be determined the AO concentration by the equation  $x=y/0.0184$ , where 'x' was the AO concentration in 32ml of sample solution and 'y' was the absorbance obtained from the UV-Visible spectrophotometric measurement. Table 3 below showed the results of AO quantification.

**Table 3:** Quantification results using UV-Visible Spectrophotometry

No.	Sample	Code	Average absorbance	Concentration (mg/kg)	Concentration (ppb)
1	Boiled bamboo shoots	S-BB-Q1	0.0087	3.008	3,008
2		S-BB-Q5	0.0153	5.281	5,281
3		S-BB-BT	0.0092	3.218	3,218
4		S-BB-TD	0.0221	7.645	7,645
		Average		4.788	4,788
5	Dried bamboo shoots	S-DB-Q4	0.0304	10.605	10,605
6		S-DB-Q5	0.0179	6.177	6,177
7		S-DB-TB	0.0449	15.598	15,598
		Average		10.793	10,793
8	Pickled cabbage	S-PC-Q4	0.0214	7.425	7,425
9		S-PC-BT	0.0436	15.165	15,165
10		S-PC-TB	0.0255	8.894	8,894
11		S-PC-TD	0.0567	19.682	19,682
		Average		12.791	12,791
Average of solid samples			0.0186	9.336	9,336

No.	Sample	Code	Average absorbance	Concentration (mg/kg)	Concentration (ppb)
12	Turmeric powder	P-TP-Q1	0.0284	9.827	9,827
13		P-TP-Q5	0.0514	17.795	17,795
14		P-TP-BT	0.0327	11.386	11,386
		<b>Average</b>		<b>13.003</b>	<b>13,003</b>
15	Curry powder	P-CP-Q4	0.0550	<b>19.081</b>	<b>19,081</b>
<b>Average of powder samples</b>			<b>0.0419</b>	<b>14.522</b>	<b>14,522</b>
<b>Total</b>		<b>Min</b>	-	<b>3.008</b>	<b>3,008</b>
		<b>Max</b>	-	<b>19.682</b>	<b>19,682</b>

AO was detected mostly in four samples of boiled bamboo shoots and four samples of pickled cabbages. The ranges were from 3.008 mg/kg to 7.645 mg/kg and from 7.425 mg/kg to 19.682 mg/kg, respectively. It was hundred thousand times higher in comparison to the amount of AO detected in the investigated boiled bamboo shoots in HCMC markets in 2016 (around 17.06 µg/kg and 3,108.94 µg/kg).

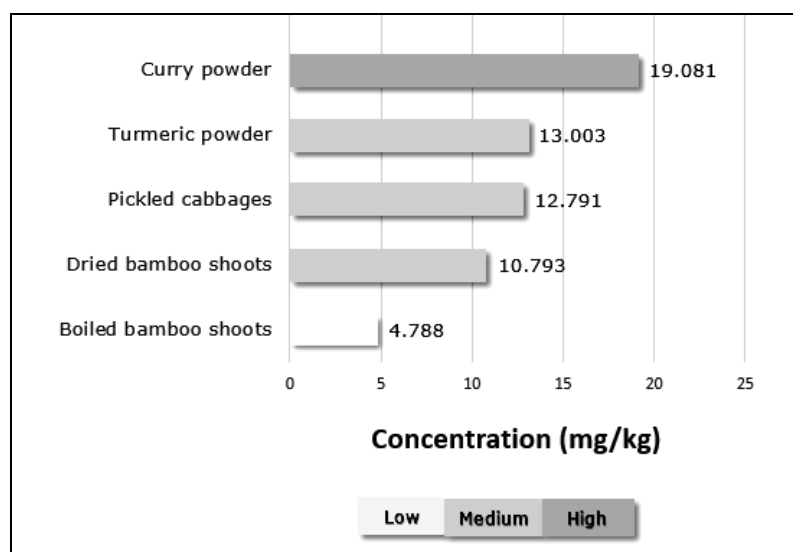
There were three dried bamboo shoots samples containing from 6.177 mg/kg to 15.598 mg/kg, which was hundred times higher than those in HCMC markets last year (around 11.84 µg/kg and 41.35 µg/kg). Turmeric powder and curry powder's contamination level were from 9.827 mg/kg to 19.081 mg/kg.

In total, the minimum and maximum AO contamination's levels were 03 mg/kg and 19 mg/kg, respectively. The average contamination amount of solid and powder food samples was 9.336 mg/kg and 14.522 mg/kg, respectively. Based on the results, it could be divided into 03 contamination levels as in Table 4.

**Table 4:** AO contamination level in collected foods

AO concentration scale (mg/kg)		3-9	9-14	14-20
Degree of contamination scale in foods		Low	Medium	High
Form	Average AO level (mg/kg)	Degree of AO contamination level		
Solid	9.336		x	
Powder	14.522			x

From Table 4, AO contamination level in solid foods was medium while it was high in powder foods. Based on Table 3 and 4, a bar chart indicating the contamination level was made. According to Fig. 6, curry powder had the highest AO contamination proportion (19.081 mg/kg in average) while boiled bamboo shoots had the lowest level of AO contamination (4.788 mg/kg in average). Turmeric powder, pickled cabbages and dried bamboo shoots contained medium level of AO (13.003 mg/kg, 12.791 mg/kg and 10.793 mg/kg, respectively).



**Figure 6:** AO contamination level in Vietnamese small scale foods

AO is an industrial dye which is banned in food in many countries including Italy, Sweden and Vietnam [4, 5]. However, its presence in food has been discovered mostly in China, India and Vietnam. The AO contamination level in Vietnamese foods, especially in bamboo shoots and pickled cabbages, has been increasing in recent years. Turmeric powder had been adulterated by using Metanil yellow for years due to its weak light stability [17]. Now it is seemed to be replaced by AO. In terms of seasoning powder category, AO concentration in turmeric powder and curry powder were nearly as equal as the result in cinnamon quills in New Zealand in 2012, which contained 15 mg/kg of AO [8].

It is a critical concern about the food safety in Vietnam. This research was still able to figure out the high rate of contamination Auramine O in local foods, and especially, the level of contamination in certain kinds of foods was much higher than what had been reported previously in public media.

## 4 CONCLUSIONS

The thin layer chromatography was appropriate for the quick detection of AO in foods. When the sample was contaminated with AO, a yellow spot would appear on the surface of the silica gel plate. Due to the yellow colour, it was difficult to observe the spot under white light. It could be clearly seen when observing under the UV light. The detection threshold was 0.06 ppm or 60 ppb.



Moreover, after the first two steps, only the mixture of AO-positive food sample was separated into 2 completely different layers: one had yellow or red transparent colour and one had opaque yellow or red colour, which depended on the original colour of the food samples. This could be considered as another rapid method to test the presence of AO in food.

There was  $12.5 \pm 3.0\%$  of a total of 120 food samples contaminated with AO, all of which belonged to a category of small-scale products from local or street markets while none of industrial ones showed the presence of AO. In terms of solid samples, there was  $15.3 \pm 4.2\%$  of random solid food samples positive to AO while the number for random powder food samples was  $8.3 \pm 4.0\%$ .

UV-Visible spectrophotometry proved to be a quick and effective method to quantify the level of AO in Vietnamese foods, which was ranged from 03 mg/kg to 19 mg/kg. 04 out of 05 kinds of food samples contained medium to high level of AO (from 09 mg/kg to 19 mg/kg) which were dried bamboo shoots, pickled cabbage, turmeric powder and curry powder. Boiled bamboo shoots contained around 4.78 mg/kg of AO in average, and it was hundred times higher than the contamination level reported previously [10].

## **5 RECOMMENDATIONS**

The research was subjected to only solid and powder food samples purchased from some local markets and supermarkets in Ho Chi Minh City, Vietnam. It would be essential to conduct the tests for liquid food or other food sources throughout the countries, especially in some rural districts or provinces. These are places far away from the cities' or provinces' downtown, which can be implied that the government management on food safety there is not considered as closed and strict.

A recommendation for consumers to wisely choose safe yellow-orange food products is that it is necessary to purchase yellow-orange foods from the supermarkets with brand-named products, which have light and more natural yellow colour. It should be avoided to choose fresh foods such as bamboo shoots, pickled cabbages or seasoning powders which have a darker and more attractive yellow colour. It is because the amount of AO contaminated in those foods are from medium to high.

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## **SESSION 3**

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# **FOOD PROCESSING AND ENGINEERING**



## QUALITY CHARACTERISTICS OF BANANA FLOUR AND STEAMED CAKE PRODUCED FROM THREE BANANA VARIETIES

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### ABSTRACT

Processing banana into flour is a way to increase its shelf life and functionality. In food industry, banana flour can be used as wheat flour substitute. In this study, banana flour and, subsequently, the banana steamed cake, were produced from three local banana varieties namely *ambon*, *kepok*, and *nangka*. Physicochemical quality attributes measured on the flour included moisture content, yield, color, water holding capacity, starch, amylose, amylopectin, fiber, and potassium content. The measurement on quality attributes of the steamed cake was texture (hardness and ease of swallowing), color, volume expansion, and potassium content. Hedonic sensory analysis was also performed for the cake. Results showed that different banana varieties gave significant differences in some parameters of the flour namely moisture, yield, color and starch content. When compared to wheat flour, banana flour had higher starch, potassium, and fiber content. Meanwhile, steamed cake quality attributes showed significant differences compared to wheat flour in terms of color, texture, and volume expansion. Hedonic test result showed that wheat flour steamed cake had the highest score but did not differ significantly from the *ambon* banana steamed cake.

**Keywords:** banana, flour, steamed-cake, variety, quality

### 1 INTRODUCTION

Banana is one of the most widely found and consumed tropical fruits in Indonesia. Banana production in Indonesia is the highest compared to other fruits such as mangoes and oranges, at up to 6.8 million tons in 2014 [1]. However, postharvest loss of banana is considered high. Therefore, there is a need to increase the shelf life as well as the commercial value of banana. One option is by converting the fruit into flour. Banana flour has low water content and water activity; hence, it will increase the shelf life [2]. Processing banana into flour also gives other benefits, such as its flexibility to be fortified with other nutrients as well as to be main ingredient of final food products [3]. Flour also make banana easier to be handled along the chains of packaging, distribution, and storage [4].

Banana flour was made from unripe banana (yellowish-green color) because ripened banana will disrupt the drying process due to its sugar content that can trigger caramelisation and contributes to the unexpected color, aroma and taste. Nevertheless, banana flour has high nutritional content. In food industries, banana flour is often used to make weaning food and bakery products [4]. Starch content in banana flour contributes to the food texture characteristics.

Many varieties of banana can be processed into flour. It was hypothesized that different banana varieties will affect to different flour characteristics. Studies by Elvis [5] and Abbas *et al* [6] reported that different banana varieties resulted in significant differences of water holding capacity, yield, and color of the flour. These can be due to the differences in physical shape, water content, and starch content of the banana [5].

Moreover, application of banana flour into food production was also reported to have different characteristics than employing wheat flour. Silfia [7] investigated brownies production applying banana or wheat flour and observed that brownies of banana flour had lower water and protein content compared to wheat flour brownies. Sensorially, taste, texture, and aroma of banana flour brownies was more preferred than wheat flour brownies. Our study applies banana flour to produce steamed cake to be compared with cake made from wheat flour.

## **2 MATERIALS AND METHODS**

### **2.1 Banana flour production**

Three local unripe (yellowish green) banana varieties namely *ambon*, *kepok*, and *nangka* were obtained from Gunungpati traditional market in Semarang. Each banana was peeled and sliced using *robot-coupe CL 50* slicer. Then, the slices were soaked in 0.2% NaS<sub>2</sub>O<sub>5</sub> solution (w/v) for 15 min. Drying was performed by a cabinet dryer at 60°C for 8 h. Dried banana slices were grinded (*Philips dry-mill blender*) and sieved at 80 mesh.

### **2.2 Steamed cake production**

Steamed cake was made by firstly mixing the dry ingredients such as flour, milk powder, and baking powder. In a different mixing bowl, eggs, sugar and emulsifier were mixed for three min. The dry mixture was added with the batter and mixed. Subsequently, melted margarine was added to the batter and mixed evenly, followed by pouring into baking tray and steaming for 25 min.

### **2.3 Physicochemical analysis**

Analyses of water content, color, and water activity were performed by using moisture balance (*Ohaus MB45*), chromameter (Minolta CR-400), and Aw meter, subsequently. Amylose content and starch content were measured by spectrophotometry method. Water holding capacity (WHC)

was measured by centrifugation method. Crude fiber content was analysed by thermogravimetric method after extraction with 0.25 N H<sub>2</sub>SO<sub>4</sub> and 0.25 N NaOH solutions. Potassium content was analysed by atomic absorption spectroscopy. Texture (hardness) was measured by a texture analyzer (*Lloyd instrument TA plus*), and volume expansion was measured by seed displacement method.

## 2.4 Hedonic test

Sensory analysis was performed by a rating hedonic test at a five-scale, by 40 SCU Food Technology Department students. Some attributes were evaluated, i.e. aroma, color, texture (in terms of hardness and ease of swallowing), taste, and overall perception.

## 2.5 Data analysis

Data obtained from the physicochemical measurements was analysed by one way ANOVA followed by Duncan test. Meanwhile, data of hedonic test was analysed by Kruskal Wallis followed by Mann Whitney test.

# 3 RESULTS AND DISCUSSION

## 3.1 Banana flour

Prior to drying, moisture content of *ambon* banana is the highest ( $66.29 \pm 2.93\%$ ) and significantly different than *kepok* banana ( $57.76 \pm 0.76\%$ ) and *nangka* banana ( $59.13 \pm 0.89\%$ ). This study used mature but unripe banana, at yellowish green color of the peel. In general, ripe banana can have higher moisture content than the unripe one. Putri [8] reported that the moisture contents of *ambon*, *nangka*, and *kepok* bananas are 73.8%, 68%, and 62%, respectively. Low moisture content and high amount of starch of unripe banana can affect to the yield of flour [4]. This study found that *ambon* banana, which contains the highest moisture content, produced the lowest yield (25%) and the *nangka* banana produced the highest yield of flour (29%).

Quality attributes of banana flour can be seen at Table 1. Moisture content of the flour is below 10% and as expected, the highest is found at *ambon* banana flour. *Nangka* banana flour has the highest yield and significantly different as compared to the lowest one. Result on water holding capacity, water activity, fiber, and starch content show that there are no significant difference between banana varieties. Meanwhile, for potassium content, the *nangka* banana flour has the highest among others.

**Table 1:** Physicochemical quality attributes of banana flour

Parameter		Banana Variety			Ref.
		<i>Kepok</i>	<i>Ambon</i>	<i>Nangka</i>	(Wheat)
Water (%)		8.35 ± 1.23 <sup>ab</sup>	9.08 ± 1.13 <sup>a</sup>	7.90 ± 0.59 <sup>b</sup>	
Yield (%)		29.18 ± 1.42 <sup>b</sup>	25.71 ± 0.26 <sup>a</sup>	31.65 ± 2.49 <sup>b</sup>	
Color	L	88.95 ± 1.54 <sup>a</sup>	90.35 ± 0.41 <sup>b</sup>	86.74 ± 1.92 <sup>c</sup>	95,54 ± 0,24 <sup>d</sup>
	a*	-0.90 ± 1.01 <sup>bc</sup>	-1.25 ± 0.55 <sup>a</sup>	-0.43 ± 0.75 <sup>c</sup>	-0,85 ± 0,07 <sup>ab</sup>
	b*	17.98 ± 2.54 <sup>b</sup>	16.28 ± 1.31 <sup>a</sup>	16.14 ± 2.76 <sup>a</sup>	9,48 ± 0,11 <sup>c</sup>
WHC (g H <sub>2</sub> O/ g)	40°C	2.77 ± 0.28 <sup>a</sup>	3.03 ± 0.38 <sup>a</sup>	2.97 ± 0.41 <sup>a</sup>	7,55 ± 0,11 <sup>b</sup>
	60°C	2.73 ± 0.24 <sup>a</sup>	2.83 ± 0.43 <sup>a</sup>	2.75 ± 0.20 <sup>a</sup>	5,67 ± 0,06 <sup>b</sup>
Aw		0.46 ± 0.01 <sup>a</sup>	0.44 ± 0.04 <sup>a</sup>	0.45 ± 0.06 <sup>a</sup>	
Fiber (%)		11.6 ± 2.4 <sup>a</sup>	12.2 ± 2.6 <sup>a</sup>	11.4 ± 2.8 <sup>a</sup>	5,3 ± 1,2 <sup>b</sup>
Starch (%)		65.71 ± 2.68 <sup>a</sup>	64.60 ± 2.66 <sup>a</sup>	67.21 ± 4.88 <sup>a</sup>	58,92 ± 0,81 <sup>b</sup>
Amylose (%)		35.27 ± 1.20 <sup>a</sup>	36.22 ± 2.94 <sup>a</sup>	34.88 ± 0.90 <sup>a</sup>	22,89 ± 0,44 <sup>b</sup>
Amylopectin (%)		64.73 ± 2.56 <sup>ab</sup>	63.78 ± 4.65 <sup>a</sup>	65.12 ± 5.16 <sup>ab</sup>	77,11 ± 0,91 <sup>b</sup>
Potassium (mg/100g)		783 ± 114 <sup>a</sup>	967 ± 154 <sup>b</sup>	988 ± 193 <sup>b</sup>	

\*) Mean  $\pm$  SD of duplicate determinations (n=9). Values with different superscripts differ significantly ( $p < 0.05$ ).

Water holding capacity (WHC) is the interaction between components in flour such as protein and starch with water in food. Our study found that the WHC of banana flour is in the range of the WHC of flour needed in the manufacturing of viscous and chewy food products such as soups, sauces, and bakery products [9]. This indicates that banana flour has good WHC to be applied in bakery products. Different temperatures were used to measure WHC, which aimed to check the gelatinization ability of each flour [10]. All banana flour show no significant changes of WHC at 40°C and 60°C, but slightly decrease for the wheat flour at 60°C. This could be due to the heating process did not reach the gelatinization temperature of banana flour, which is 68.8-77.5°C, while the gelatinization temperature of wheat flour is 53-63°C [7]. Another possible explanation is due to different protein content. One of the factors affecting WHC is protein



because it can absorb and bind water, higher protein content will impact to higher binding water ability [11].

The low water activity of the flour (0.44-0.46) could prevent growth of microorganisms and reduce the rate of rancidity of flour [12]. Banana flour also contains higher fiber content than wheat flour. Referring that fiber requirement for adults per day is  $\pm 30$ g, depending on total calorie intake [13], intake of 100 g of these banana flour can provide 30% of fiber. Moreover, potassium is one of the essential minerals to control total volume of body fluids, acids and electrolytes, as well as maintain cell function. Potassium contents in the banana flour are 0.783-0.988%. The daily nutritional intake recommended by WHO for potassium is 3510 mg/day for adults [14]. This indicates that 100 grams of banana flour can fulfilled 22-28% potassium needs per day.

### 3.2 Steamed cake

Quality attributes of steamed cake produced from banana flour and wheat flour are shown in Table 2. Wheat cake has the brightest appearance while *ambon* banana cake is the darkest. The wheat cake is also more yellow than others. Apparently, this is due to the color of the flour; whereas wheat flour is white and banana flour is light brown. All banana steamed cakes show no significant difference in volume expansion but the wheat cake is significantly the highest. This could be due the role of protein in wheat in entrapping gas formed during mixing and contribute to dough viscosity and extensibility [15].

In addition, hardness level of wheat cake is the highest. Texture level of the cake is correlated with the WHC of the flour, i.e. higher WHC is followed by harder texture. This is probably due to the smaller size of starch granule in wheat flour (2-35  $\mu$ m) than banana flour (60-105  $\mu$ m). Large starch granules can cause bonded water molecules to be easily released during heating or cooking process. The more water is released during heating, the softened the product could be obtained [7].

**Table 2:** Physicochemical quality attributes of steamed cake

Parameter		Type of Flour*			
		<i>Kepok</i>	<i>Ambon</i>	<i>Nangka</i>	Wheat
Color	L	63,08 $\pm$ 2,27 <sup>bc</sup>	61,01 $\pm$ 1,60 <sup>b</sup>	66,53 $\pm$ 2,84 <sup>c</sup>	82,01 $\pm$ 2,81 <sup>a</sup>
	a*	1,15 $\pm$ 0,09 <sup>c</sup>	1,86 $\pm$ 0,11 <sup>b</sup>	0,39 $\pm$ 0,20 <sup>d</sup>	-4,59 $\pm$ 0,16 <sup>a</sup>
	b*	17,49 $\pm$ 0,3 <sup>b</sup>	17,06 $\pm$ 1,54 <sup>b</sup>	18,92 $\pm$ 0,84 <sup>b</sup>	31,36 $\pm$ 1,51 <sup>a</sup>
Hardness (gf)		158,18 $\pm$ 3,39 <sup>b</sup>	167,45 $\pm$ 7,17 <sup>bc</sup>	196,53 $\pm$ 4,83 <sup>c</sup>	319,53 $\pm$ 8,88 <sup>a</sup>

Parameter	Type of Flour*			
	<i>Kepok</i>	<i>Ambon</i>	<i>Nangka</i>	Wheat
Volume Expansion (%)	48,51 ± 0,20 <sup>a</sup>	48,78 ± 0,58 <sup>a</sup>	48,85 ± 0,33 <sup>a</sup>	53,95 ± 0,99 <sup>b</sup>
Potassium (mg/100g)	44,9 ± 3,8 <sup>a</sup>	53,8 ± 2,4 <sup>b</sup>	58,5 ± 0,8 <sup>c</sup>	-

\*) Mean ± SD of duplicate determinations (n=9). Values with different superscripts differ significantly ( $p < 0.05$ ).

Hedonic test that was performed by 40 respondents shows that *kepok* flour cake is the least liked than others (Table 3). The most liked cake is the wheat flour cake; but, it has no significant difference with *nangka* banana cake.

**Table 3:** Hedonic rate on the steamed cakes

Type of Flour	Parameter*				
	Aroma	Color	Texture	Taste	Overall
<i>Kepok</i>	2,48±1,24 <sup>b</sup>	2,78±0,95 <sup>bc</sup>	3,25±0,90 <sup>a</sup>	2,95±0,93 <sup>c</sup>	2,80±0,82 <sup>b</sup>
<i>Ambon</i>	3,55±0,85 <sup>a</sup>	2,88±0,94 <sup>b</sup>	3,63±0,84 <sup>a</sup>	3,50±0,91 <sup>ab</sup>	3,45±0,68 <sup>a</sup>
<i>Nangka</i>	3,20±1,18 <sup>a</sup>	2,35±0,98 <sup>c</sup>	3,05±0,96 <sup>a</sup>	3,20±0,97 <sup>bc</sup>	3,03±0,83 <sup>b</sup>
Wheat	3,55±1,11 <sup>a</sup>	3,68±1,19 <sup>a</sup>	3,40±1,03 <sup>s</sup>	3,73±0,75 <sup>a</sup>	3,73±0,78 <sup>a</sup>

\*) Mean ± SD of duplicate determinations (n=9). Values with different superscripts differ significantly ( $p < 0.05$ ).

For color, panelists preferred the color of wheat flour cake the most. Meanwhile, for texture (hardness and ease of swallowing), there is no significant difference of preference between samples. For aroma and taste, wheat flour cake achieves the highest score compared to *kepok* and *nangka* banana cakes but has no significant difference with *ambon* banana cake. When judging the overall attributes, the steamed cakes made of *ambon* banana and wheat flour are the most preferred by respondents.

## 4 CONCLUSIONS

Among three banana varieties, *nangka* banana flour produces higher yield, starch, and potassium content, while *ambon* banana flour has brighter color and higher fiber content. For steamed cakes, the wheat flour cake has brighter appearance, harder texture and higher volume expansion. For overall attribute of sensory, there is no significant difference between wheat and *ambon* banana cakes in term of hedonic rating.

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## ANTHOCYANIN RETENTION IMPROVEMENT OF MICROENCAPSULATED BUTTERFLY PEA FLOWER CRUDE EXTRACT BY USING FREEZE DRYING AND $\beta$ -CYCLODEXTRIN

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### ABSTRACT

Butterfly pea flower crude extract has been used as natural colorant in foods since the ancient time. Many encapsulation techniques, such as spray drying and freeze drying, have been used to increase its stability. However, the retention of the compounds has not yet improved. Therefore, this study was aimed to optimize the encapsulation of butterfly pea extract by using freeze drying and  $\beta$ -cyclodextrin to improve the anthocyanin retention. The stability of the powder was also investigated. Chemical and physical properties of encapsulated powders, such as moisture content, total anthocyanin contents as well as encapsulation yield (EY) and encapsulation efficiency (EE) were analyzed. The ratio of the extract to the wall materials (75% maltodextrin and 25%  $\beta$ -cyclodextrin) as 1:1 and freeze-drying method could retain the highest anthocyanin content as high as 88.4% with good color profile ( $L^* = 38.62$ ,  $a^* = 2.67$ , and  $b^* = -21.07$ ). An increasing of the wall material proportion and  $\beta$ -cyclodextrin tended to cause the color fading and reduced the anthocyanin content in the powder. Moreover, the storage stability test was investigated at 35, 45, and 45°C and  $a_w$  of 0.53, 0.64, and 0.75 for 5 weeks. In each week, the quality of the encapsulated powder was evaluated. There were the significant effects of temperature and interaction of temperature and water activity on the first order kinetic reaction of anthocyanin. The most suitable condition of storage was 45°C with  $a_w$  of 0.53 and 0.64, providing the kinetic constant (k) of 0.1026 and 0.0881; and correlation coefficient ( $R^2$ ) of 0.9806 and 0.9906 with the half-life of 6.76 and 7.93 weeks for  $a_w$  of 0.53 and 0.64, respectively.

**Keywords:** anthocyanin, microencapsulation, cyclodextrin, freeze drying, maltodextrin

### 1 INTRODUCTION

The most striking feature of butterfly pea (*Ciliteria ternatea*), a perennial climber found commonly in Southeast Asia, is its deep blue flower. It has held the potential to be used as a natural colorant applied in foods and cosmetics [1], [2]. The deep blue color of flower is associated with polyacylated anthocyanins pigment characterized as acylated delphinidin 3,

3'5'tri-glucosides. The properties of anthocyanins provide the solubility and beautiful hue; however, it also brings about the instability problem. The pigments are remarkably vulnerable and highly susceptible to degradation by various factors, including their chemical structure, pH, temperature, enzyme, light, oxygen, and other accompanying substances such as ascorbic acid, sugars, sulfur dioxide, and metallic ions [3]. When the structure is disrupted, it becomes colorless or brown colored compound. Consequently, this phenomenon restricts the utilization of the anthocyanin colorant. Encapsulation is the promising technology to help protecting sensitive ingredient as anthocyanins from surrounding by embedding the molecule inside of the barrier material, and the component is released when they are desired. There are various matrixes that have been used to incorporate the active agents and cyclodextrin is one of them.  $\beta$ -cyclodextrin ( $\beta$ -CD) is one of a group of cyclic oligosaccharides consisting of six or more glucose units. This compound can complex with molecules via its unique structure. Many studies applied this compound in trapping the anthocyanin and found to ameliorate the stability. Tantituvanont et al. [4] conducted the research on the effect of  $\beta$ -CD on thermal stability of the butterfly pea extract, which found to improve the samples in both solution and solid state. It also improved the retention of the anthocyanin in chokeberry juice at pH 3.6 after 8 [5]. Moreover, freeze drying is of interest for encapsulation of butterfly pea flower crude extract due to low temperature used to avoid the degradation of anthocyanin by heat, leading to better anthocyanin retention. Therefore, this research was aimed to improve anthocyanin retention by using  $\beta$ -CD and freeze drying for encapsulation of butterfly pea flower crude extract. The stability of encapsulated powder was also investigated through 5 weeks storage.

## **2 MATERIALS AND METHODS**

### **2.1 Preparation of butterfly pea extract**

The flowers of butterfly pea were purchased from the commercial market. After removing stigmas and anther, petals were dried at 45°C for 24 hours. Dried petals were cut into small pieces and soaked in distilled water in ratio of 1:40 for 3 hours. The suspension was filtered through a filter paper (Whatman no.1), and the solution was concentrated by using rotary evaporator until the concentrate reached  $16 \pm 0.5\%$  soluble solid content.

### **2.2 Encapsulation process**

The carrier agent Maltodextrin 20DE (Corn Products, Thailand) and  $\beta$ -CD (Xi'an Hong Chang Pharmaceuticals, China) were combined with the pigment extract as shown in Table 1 and stirred until all the materials were completely dissolved. Subsequently, the mixtures were homogenized at 600 rpm for 15 minutes. The resulting solutions were dried by using freeze dryer (at -35°C and 0.05 mbar) for 24 hours and then the extracts cakes were converted into powder.

**Table 1:** Formulation with different portion and composition of encapsulating agent

Treatment	Portion of core to wall material	Encapsulating agent	
		β-CD	MD
1	1:1	25	75
2		50	50
3		75	25
4	1:2	25	75
5		50	50
6		75	25
7	1:3	25	75
8		50	50
9		75	25

### 2.3 Stability evaluation of the encapsulated butterfly pea extract

Encapsulated powders were stored at 3 different temperatures (35°C, 45°C, and 55°C) and 3 different  $a_w$  (0.53, 0.64, and 0.75, by using desiccator containing a saturated solution of 11 M NaCl, NaNO<sub>2</sub> and NaCl, respectively) [5]. The degradation was monitored for 5 weeks and chemical and physical analyses were conducted weekly. The half-life of the samples was determined as well [6].

### 2.4 Encapsulation yield (EY)

*The process yield is calculated using following formula:*

$$EY (\%) = \frac{\text{weight of the encapsulated powder}}{\text{Total weight of all ingredients}} \times 100$$

### 2.5 Encapsulation efficiency (EE)

The Encapsulation efficiency is calculated using following formula:

$$EE (\%) = \frac{\text{Total anthocyanin content in powder}}{\text{Total anthocyanin in extract}}$$

## 2.6 Chemical and physical analysis

### 2.6.1 Moisture content

Weighted samples (1.0 g) were oven dried at 105°C for 8 hours in pre-weighed moisture cans. The moisture cans were transferred to desiccators immediately, cooled and weighed. The moisture content was then calculated as follow:

$$\text{Moisture (\%)} = \frac{\text{Loss in weight (g)}}{\text{Sample weight (g)}} \times 100$$

### 2.6.2 Total anthocyanin content

Total anthocyanin content (TAC) was evaluated using pH-differential method described by Lee et al. [8]. The test samples were diluted with potassium chloride (pH 1.0) and sodium acetate (pH 4.5). The absorbance was measured at 520 and 700 nm against the distilled water filled in a blank cell. The results were expressed as mg of cyanidin-3-glucoside of 100 g of dry matter of sample, which calculated from following formula:

$$\text{Anthocyanin pigment} = \frac{A \times MW \times DF \times 10^3}{\epsilon \times 1}$$

Where  $A = (A_{520} - A_{720})_{\text{pH}1.0} - (A_{520} - A_{700})_{\text{pH}4.5}$ ; molecular weight (MW) = 440.2 g/mol; DF is the dilution factor, extinct coefficient ( $\epsilon$ ) = 26,900 L cm<sup>-1</sup> mol<sup>-1</sup>. Triplicate analysis was performed for each treatment.

### 2.6.3 Colorimetric analysis

The color of encapsulated powders were determined as L\*, a\*, and b\* values using a HunterLab Miniscan EZ in triplicate. It was used to investigate all samples after freeze drying and during 5-week stability test. Moreover, in the stability test, total color differences ( $\Delta E$ ) was calculated by this following equation:

$$\Delta E_{ab}^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

$\Delta L^*$  ( $L^*$  sample minus  $L^*$  standard) = difference in lightness and darkness (+ = lighter, - = darker),  $\Delta a^*$  ( $a^*$  sample minus  $a^*$  standard) = difference in red and green (+ = redder, - = greener),  $\Delta b^*$  ( $b^*$  sample minus  $b^*$  standard) = difference in yellow and blue (+ = yellower, - = bluer).

### 2.6.7 Statistical analysis

A randomized block design and 3×3 factorial designs with 3 replications were used. Statistical analysis of measured results was carried out by using ANOVA procedure, and significant



differences were determined by using Duncan's multiple range test ( $p \leq 0.05$ ) by IBM SPSS Statistics 20.0. Moreover, one sample t test was conducted to compare the difference between two samples.

### **3 RESULTS AND DISCUSSION**

#### **3.1 Encapsulation yield (EY)**

The process yield (Table 2) was not affected by the difference concentrations of  $\beta$ -CD and its interaction with proportion of wall material. It was positive correlated with the proportion of core and wall materials. The highest yield (72.7%) was found at ratio 1:3 and 25%  $\beta$ -CD concentration and lowest yield (47.3%) was found at ratio 1:1 and 25%  $\beta$ -CD concentration. It might result from the body provided by the wall material. When the amount of the body increased, the yield of the powder obtained also increased. However, the lowest yield of the experiment was about 12% greater than the highest production yield (35.2%) of encapsulated butterfly pea using spray drying technique [4]. Freezing drying technique may prevent the loss that occurred in spray drying including the loss of fine particles as well as the residue left in the dryer or cyclone.

#### **3.2 Encapsulation efficiency (%EE)**

The encapsulation efficiency of all samples was in the range of 11.14 - 88.41%, in which both ratios of core/wall materials and  $\beta$ -CD/maltodextrin were significant affected ( $p \leq 0.05$ ). From Table 2, it can be observed that the increase in wall material in the mixture tended to decrease %EE. The EE of 50%  $\beta$ -CD at 1:1 ratio was higher than that of 1:2 and 1:3 approximately 2 and 3 times, respectively. This might be the result from the lesser extract added and less distribution of the extract throughout the matrix.

Concerning the  $\beta$ -CD concentration, it also had negatively influenced on EE as well. For instance, regard to 1:1 ratio, when  $\beta$ -CD concentration increased from 25 to 50%, the anthocyanin content declined from 1126.0 to 690.8 mg/100 g of dry matter content, or approximately 34% EE; while increasing from 50 to 75% of BCD concentration, % EE was reduced by 50% or approximately 325.7 mg/100 g of dry matter content. Of these, there might be some interaction between the anthocyanin and  $\beta$ -CD that led to the decline of % EE in the presence of  $\beta$ -CD. It has been found that  $\beta$ -CD was favored to interact with the colorless chalcone form, and it also catalyzed the structural transformation of callistephin (pelargonidin-3-O-glucoside) and chrysanthemin (cyanidin-3-O-glucoside), shifting the pigment hydration equilibrium toward the formation of more colorless anthocyanin. Higher concentration of  $\beta$ -CD induced greater effect [9]. Moreover, Frenandes et al. [10] used <sup>1</sup>H NMR spectra to observe anthocyanin betacyclodextrin complex and described that there was a disruption of self-association of anthocyanin structure, which brought about the decrease in concentration.

The highest % EE among all 9 samples is the formula that has least matrix proportion and least  $\beta$ -CD in the mixture (ratio of the extract to the wall material containing 25% BCD concentration). It

was shown that the same portion of core and wall material was sufficient to trap the anthocyanins of the butterfly extract and provided the highest EE of 88.41%. Besides, the effect of high matrix portion and high  $\beta$ -CD concentration made EE lessen to 11.14%.

**Table 2:** Some chemical and physical properties of encapsulated butterfly pea flower extract

Portion of core to wall material	Concentration of $\beta$ -CD	Yield (%)	TAC (mg/100g of dry matter)	EE (%)	Moisture content (%)	L*	a*	b*
1:1	25%	47.3 $\pm$ 3.18 <sup>a</sup>	1126.0 $\pm$ 29.58 <sup>a</sup>	88.4 $\pm$ 2.32 <sup>a</sup>	7.4 $\pm$ 0.31 <sup>bc</sup>	38.62 $\pm$ 4.96 <sup>a</sup>	2.67 $\pm$ 0.10 <sup>a</sup>	-21.07 $\pm$ 0.79 <sup>a</sup>
	50%	51.2 $\pm$ 2.63 <sup>c</sup>	690.8 $\pm$ 5.63 <sup>b</sup>	54.2 $\pm$ 0.44 <sup>b</sup>	8.0 $\pm$ 0.18 <sup>d</sup>	44.71 $\pm$ 2.21 <sup>b</sup>	2.50 $\pm$ 0.10 <sup>ab</sup>	-20.13 $\pm$ 0.75 <sup>ab</sup>
	75%	48.9 $\pm$ 0.26 <sup>c</sup>	325.7 $\pm$ 3.81 <sup>c</sup>	25.6 $\pm$ 0.30 <sup>c</sup>	7.7 $\pm$ 0.13 <sup>cd</sup>	50.16 $\pm$ 2.46 <sup>bc</sup>	0.88 $\pm$ 0.10 <sup>d</sup>	-18.08 $\pm$ 0.45 <sup>cd</sup>
1:2	25%	63.7 $\pm$ 0.01 <sup>b</sup>	363.9 $\pm$ 13.56 <sup>c</sup>	28.6 $\pm$ 1.06 <sup>c</sup>	7.3 $\pm$ 0.25 <sup>b</sup>	50.43 $\pm$ 2.37 <sup>bc</sup>	2.21 $\pm$ 0.14 <sup>b</sup>	-19.07 $\pm$ 0.02 <sup>bc</sup>
	50%	63.5 $\pm$ 0.03 <sup>b</sup>	342.1 $\pm$ 13.84 <sup>c</sup>	26.9 $\pm$ 1.09 <sup>c</sup>	7.7 $\pm$ 0.09 <sup>cd</sup>	56.20 $\pm$ 2.60 <sup>cd</sup>	1.45 $\pm$ 0.40 <sup>c</sup>	-18.47 $\pm$ 0.31 <sup>cd</sup>
	75%	62.2 $\pm$ 1.54 <sup>b</sup>	227.1 $\pm$ 6.37 <sup>de</sup>	17.8 $\pm$ 0.55 <sup>de</sup>	7.3 $\pm$ 0.24 <sup>b</sup>	57.40 $\pm$ 3.10 <sup>d</sup>	0.33 $\pm$ 0.15 <sup>e</sup>	-17.62 $\pm$ 0.15 <sup>cd</sup>
1:3	25%	72.7 $\pm$ 2.67 <sup>a</sup>	267.0 $\pm$ 36.10 <sup>d</sup>	21.2 $\pm$ 2.83 <sup>d</sup>	6.7 $\pm$ 0.31 <sup>a</sup>	55.91 $\pm$ 0.03 <sup>cd</sup>	1.70 $\pm$ 0.04 <sup>c</sup>	-16.82 $\pm$ 0.04 <sup>de</sup>
	50%	72.0 $\pm$ 2.00 <sup>a</sup>	219.9 $\pm$ 8.42 <sup>e</sup>	17.3 $\pm$ 0.66 <sup>c</sup>	6.8 $\pm$ 0.09 <sup>a</sup>	58.26 $\pm$ 0.72 <sup>d</sup>	1.50 $\pm$ 0.01 <sup>c</sup>	-15.96 $\pm$ 0.01 <sup>e</sup>
	75%	69.0 $\pm$ 1.11 <sup>a</sup>	141.9 $\pm$ 18.37 <sup>f</sup>	11.1 $\pm$ 1.44 <sup>f</sup>	6.7 $\pm$ 0.32 <sup>a</sup>	58.36 $\pm$ 5.46 <sup>d</sup>	0.40 $\pm$ 0.12 <sup>e</sup>	-15.31 $\pm$ 0.12 <sup>e</sup>

\*Means within a column with different letters are significantly different ( $p \leq 0.05$ ).

### 3.3 Some chemical and physical of properties of encapsulated butterfly pea flower anthocyanin

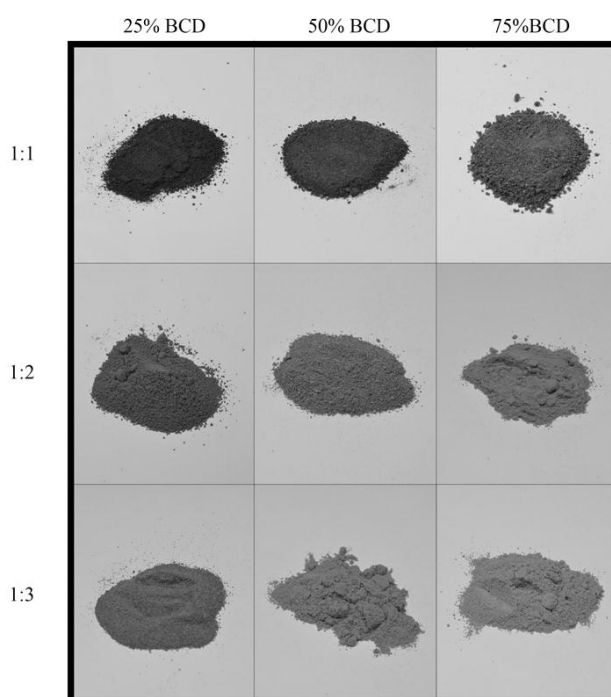
#### 3.3.1 Moisture content

The moisture content of the encapsulated powder is presented in Table 2. It varied from 6.72 - 8.0% which was significantly influenced by the portion of core and matrix ( $p \leq 0.05$ ), whereas there was no effect from the concentration of  $\beta$ -CD and its correlation with proportion of matrix ( $p > 0.05$ ). The greater proportion of wall materials provided lower moisture content. Therefore, with the highest ratio of 1:3, it gave the lowest moisture content of 6.72% for 25% and 50%  $\beta$ -CD and 6.78% for 75%  $\beta$ -CD. The moisture contents of encapsulated powder were not different to encapsulation of butterfly pea flower by spray drying method using HPMC/gelatin as wall materials and freeze-drying method [4]; maltodextrin/maltodextrin and algenate/algenate [9]. Their moisture contents ranged from 5.5 - 8.3% and 6.5 - 7.2%, respectively. However, vacuum oven drying method provided lower moisture content (3.5 - 5.7%).

#### 3.3.2 Color characteristics

The color of encapsulated powder was measured by using HunterLab Model, which gives the result of three parameters including L\* and two coordinates a\* and b\*. L\* refers as the lightness that minimum value representing black is 0 and maximum value representing white is 100. Positive a\* value indicates the direction of redness whereas negative a\* value indicates

greenness. For  $b^*$ , yellowness is in the negative range and blueness is in the positive range. According to the result of all three parameters in Table 2, they were influenced by both wall material portion and  $\beta$ -CD quantity in the mixture ( $p \leq 0.05$ ). The portion made the difference in  $L^*$  providing 1:2 and 1:3 ratio lighter than 1:1, whereas the increased  $\beta$ -CD to 50 and 75% significantly yielded higher  $L^*$  value. It was possible that the formula containing high quantity of wall material was lighter due to limited extract for trapping and well distribution. In addition, the interaction of anthocyanin  $\beta$ -CD complex did not only affect the anthocyanin content trapped in the molecules but also the fading of the color. Lewis and Walker [11] reported the decrease of anthocyanin color in both pH 2 and 4 in the presence of  $\beta$ -CD and the effect was increased with the increased concentration, whereas Dangles et al. [12] reported the fading color of callistephin due to  $\beta$ -CD as the anti-copigment effect.



**Figure 1:** Encapsulated powders of butterfly pea flower extract using the mixture of maltodextrin and  $\beta$ -cyclodextrin and freeze-drying method

In accordance to the positive  $a^*$  and negative  $b^*$  shown in Table 2, all encapsulated powder samples exhibited the color ranging from purplish blue to dark slate blue. The variation occurred due to ratio of matrix and  $\beta$ -CD concentration, which was believed to cause by the same phenomena as  $L^*$ . The high amount of matrix had less extract to be trapped in the molecules, whereas the complexation with anthocyanin changed the blue color of butterfly pea to colorless.

From Fig. 1, it can be seen that color of butterfly pea samples was in corresponding to the result of  $L^*$ ,  $a^*$ , and  $b^*$ . The color faded down from dark blue to light slate blue when matrix portion increased and  $\beta$ -CD increased.

Based on all qualities, the formulation that had a ratio of core to wall material at 1:1 and 25%  $\beta$ -CD was the best condition. It encapsulated the highest anthocyanin content in the powder, with 88.4 % EE, and provided the dark slate blue with less color fading than other samples.

Comparing to control (0%  $\beta$ -CD) and the ratio of core and matrix as 1:1, it was found that there were no significant differences on all attributes ( $p > 0.05$ ), shown in Table 3. Although  $\beta$ -CD may cause the color diminishing effect or decreasing anthocyanin content, adding 25%  $\beta$ -CD does not remarkably affect the qualities of encapsulated powder. The complexation might also lead to the anthocyanin stabilization [9].

**Table 3:** Color properties of butterfly pea flower extract produced by using spray drying/maltodextrin (Commercial) and freeze drying/maltodextrin- $\beta$ -CD (Sample) and freeze drying/maltodextrin (Control)

Sample	TAC (mg/100g of dry matter)	L*	a*	b*
Control	1206.4 $\pm$ 27.85 <sup>a*</sup>	35.48 $\pm$ 3.78 <sup>ab</sup>	3.10 $\pm$ 0.15 <sup>a</sup>	-21.28 $\pm$ 0.67 <sup>a</sup>
Sample	1126.0 $\pm$ 29.58 <sup>a</sup>	38.62 $\pm$ 4.96 <sup>a</sup>	2.67 $\pm$ 0.10 <sup>a</sup>	-21.07 $\pm$ 0.79 <sup>a</sup>

\*Means within a column with different letters are significantly different ( $p > 0.05$ ).

### 3.4 Stability test

Stability of the microencapsulated butterfly pea extracts was evaluated during 5 weeks in three different temperatures (35, 45, and 55°C) and three different water activity levels (0.53, 0.64, and 0.75). In each week the samples were taken and analyzed. Table 4 summarized the results including half-life and all other parameters of the 5-week of the stability test.

For half-life, since many researches supported that the degradation of anthocyanin during the storage is the first order reaction [13], [14]; therefore, the determination of half-life of encapsulated butterfly pea flower extract was based on the first order reaction. According to Table 4 it was noticed that the encapsulated powder had half-life between 4.51-7.93 weeks, which was mainly influenced by temperature and its correlation with water activity ( $p \leq 0.05$ ). For example, the half-life of encapsulated powder stored at 55°C/ $a_w$  0.53 was less than that of at 35°C and 45°C about 2 weeks. Moreover, the interaction effect of water activity and temperature provided the longest half-life of temperature of 55°C at  $a_w$  0.75 and decreasing  $a_w$  reduced the storage time. Jimenez et al. [15] reported that the reduction in water activity from 0.99 to 0.34 at high temperature (100-140°C) increased the degradation rate of anthocyanin 4 folds. It resulted from polymerization reaction that favored to occur at low water activity. It is proposed that the same phenomenon might occur at 55°C when stored in a long time as well. Overall, the conditions that provide the highest half-life was at 35°C with  $a_w$  0.53 and 0.75 and at 45°C with  $a_w$  0.53 and 0.64.

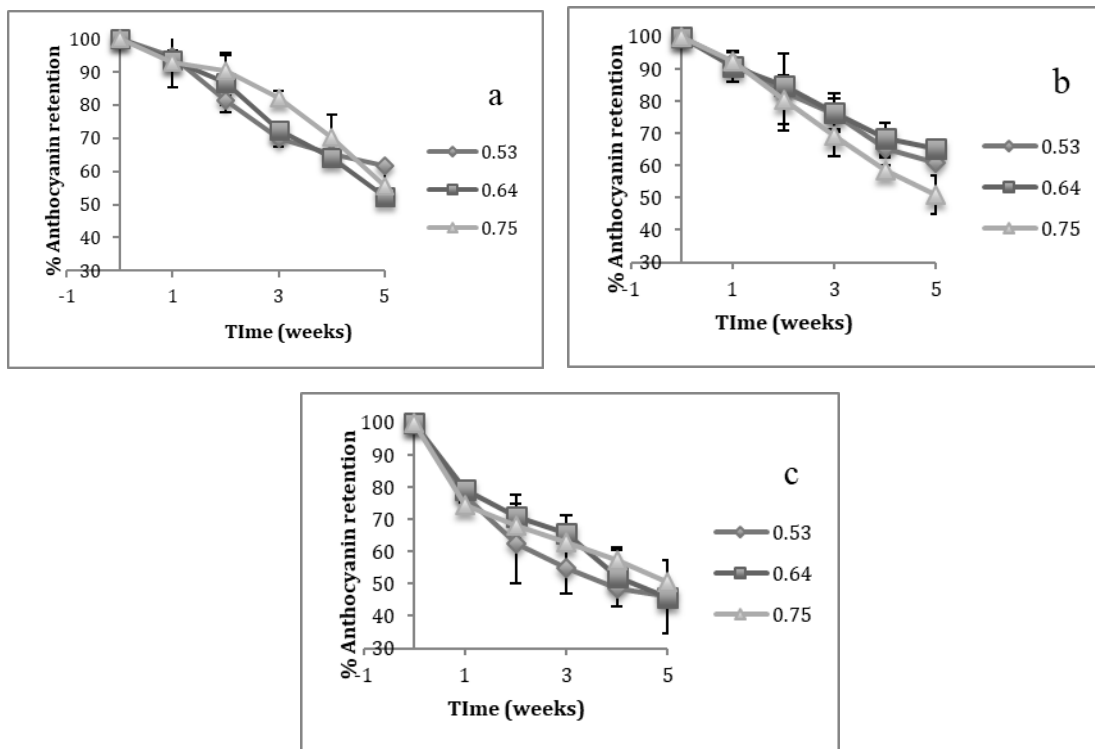
The changes in anthocyanin retention over 5-week stability test are shown in Fig. 2. Fig. 2 shows the effect of temperature on anthocyanin retention over time. It was found that temperature has a negative effect on anthocyanin, and higher heat intensity causes greater effect on its degradation. The influence of this factor has been found to cause the impact and it has been suggested to possibly cause by the destruction of glycosidic bond, which brings about the instability, or heat induced ring opening, converting the pigment to colorless form. Roobha et al. [16] reported that anthocyanin pigments in *Musa acuminata* bract was stable at low temperature and rapidly destructed at 40°C and above, whereas the anthocyanin of grape juice degraded higher with the rise of temperature [17]. In contrast, the water activity seemed not to have an influence on the retention as shown in Fig. 2. Trends of three water activity levels were similar; however, the interaction of water activity and temperature was found. The anthocyanin content at 45°C and  $a_w$  0.75 decreased more sharply than other two water activity levels. Therefore, it could be concluded that temperature affected on the retention of anthocyanin and increasing water activity up to 0.75 caused the greater destruction rate at 45°C. Moreover, Table 4 indicated that the highest anthocyanin retention conditions were 35°C with  $a_w$  0.53 and 0.75; and at 45°C with  $a_w$  0.53 and 0.64, which were similar to half-life results.

**Table 4:** Degradation kinetic of anthocyanin degradation of encapsulated powder and color quality evaluation at the end of storage test

Temp. (°C)	$a_w$	1 <sup>st</sup> order reaction			$L^*$	$a^*$	$b^*$	$\Delta E$	% Moisture content
		$t_{1/2}$ (weeks)	$K$ (week <sup>-1</sup> )	$R^2$					
35	0.53	6.59 <sup>abc*</sup>	0.1052±0.0002	0.9677	61.7±0.5 <sup>ab</sup>	33.11±1.20 <sup>a</sup>	2.13±0.51 <sup>a</sup>	-12.89±1.60 <sup>bc</sup>	12.8±0.5 <sup>c</sup>
	0.64	5.32 <sup>bcd</sup>	0.1304±0.0002	0.9557	52.3±1.9 <sup>bc</sup>	36.67±0.72 <sup>ab</sup>	1.60±0.61 <sup>d</sup>	-11.99±0.99 <sup>d</sup>	11.9±0.3 <sup>c</sup>
	0.75	6.32 <sup>abc</sup>	0.1109±0.0002	0.8672	55.6±5.9 <sup>abc</sup>	37.94±0.17 <sup>bc</sup>	1.63±0.18 <sup>cd</sup>	-12.65±0.28 <sup>c</sup>	11.9±0.3 <sup>c</sup>
45	0.53	6.76 <sup>ab</sup>	0.1026±0.0028	0.9548	60.8±0.3 <sup>ab</sup>	38.12±1.30 <sup>bc</sup>	1.86±0.28 <sup>bc</sup>	-12.82±0.55 <sup>bc</sup>	8.4±0.3 <sup>b</sup>
	0.64	7.93 <sup>a</sup>	0.0881±0.0110	0.9716	65.4±2.8 <sup>a</sup>	39.35±1.58 <sup>bcd</sup>	1.83±0.05 <sup>cd</sup>	-13.33±0.11 <sup>ab</sup>	8.0±0.1 <sup>ab</sup>
	0.75	4.95 <sup>cd</sup>	0.1405±0.0101	0.9519	50.9±5.9 <sup>bc</sup>	36.67±0.30 <sup>ab</sup>	2.08±0.17 <sup>ab</sup>	-13.67±0.50 <sup>a</sup>	8.5±1.2 <sup>b</sup>
55	0.53	4.51 <sup>d</sup>	0.1537±0.0005	0.9103	46.2±2.2 <sup>c</sup>	40.71±0.39 <sup>cd</sup>	0.01±0.78 <sup>e</sup>	-0.70±0.70 <sup>g</sup>	7.4±0.7 <sup>ab</sup>
	0.64	4.85 <sup>cd</sup>	0.1525±0.0539	0.9178	45.8±11.3 <sup>c</sup>	43.01±3.47 <sup>d</sup>	0.02±0.17 <sup>e</sup>	-3.85±0.91 <sup>e</sup>	6.9±0.4 <sup>a</sup>
	0.75	5.68 <sup>bcd</sup>	0.1220±0.0046	0.9208	50.7±0.4 <sup>bc</sup>	40.37±0.30 <sup>bcd</sup>	0.11±0.46 <sup>e</sup>	-1.41±0.34 <sup>f</sup>	7.4±0.1 <sup>ab</sup>

\*Means within a column with different letters are significantly different ( $p \leq 0.05$ ).

$R^2$  is the relative coefficient.



**Figure 2:** The effect of temperature and water activity on anthocyanin retention during 5-week stability test a) 35°C, b) 45°C and c) 55°C

The color quality was monitored by three parameters ( $L^*$ ,  $a^*$ , and  $b^*$ ) whereby the average initial color attributes were  $L^* = 38.14$ ,  $a^* = 2.43$ , and  $b^* = -17.80$ . The impact of temperature on the lightness ( $L^*$ ) can be observed in Figure 3. It shows that each temperature has a marginal difference in trend of lightness change. At 35°C  $L^*$  decreased until week 4 and raised up in week 5, which might be due to degradation of anthocyanin, whereas at 45°C the increasing of  $L^*$  started at week 3. For 55°C,  $L^*$  was slightly steady at the beginning except for  $a_w$  0.53 that  $L^*$  moderately declined. However, in the last three weeks, the lightness of all samples hiked up. Moreover, comparing the increasing and decreasing ranges of  $L^*$ , it was found that lower temperature tended to give lower  $L^*$  while higher temperature gave higher  $L^*$ . In Figure 3, it can be observed that water activity did not cause the effect on the change of  $L^*$  value but it had a particular interaction in reducing of  $L^*$  at  $a_w$  0.75/35°C (Fig. 3). Therefore, the temperature was positive correlated with the lightness, and some interaction with  $a_w$  might cause increasing and decreasing of  $L^*$ . Accordingly,  $L^*$  of the fifth week also elaborated the effect of the factors.

$a^*$  value as well as  $b^*$  value was reduced during 5-weeks, which indicated the degradation of anthocyanin content and the loss of encapsulated powder's blue color.  $a^*$  was influenced by temperature as seen in Fig. 3. When the temperature increased,  $a^*$  tended to reduce markedly. Especially at 55°C,  $a^*$  declined dramatically within the first week and continue reducing steadily down to 0.01-0.02, and 0.11 at  $a_w$  0.53, 0.64, and 0.75, respectively in the fifth week. For water

activity, it negatively affected to  $a^*$  only at 35°C whereas other temperatures caused no effect. Regarding to  $b^*$  value, the high temperature (55°C) caused the reduction of  $b^*$  value vastly, giving the lowest  $b^*$  at the end whereas the decline of  $b^*$  value in other two temperatures were slightly different from each other. On the other hand, the water activity caused the difference in change of  $b^*$  over time at 55°C.

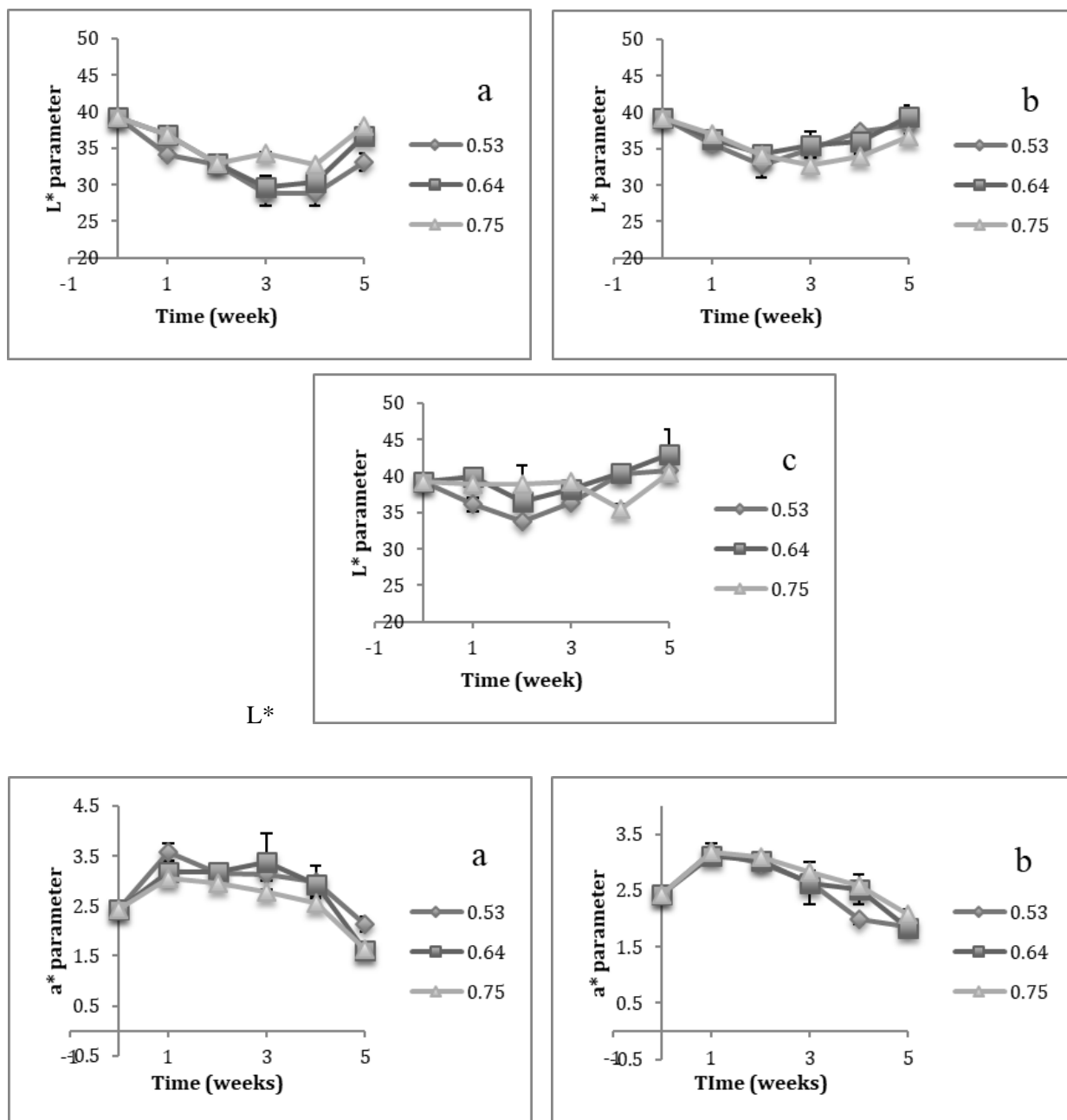
$\Delta E$  was calculated to identify the total color difference among all nine conditions. The data was reported in Table 4 indicating that the lowest change of color was found at 35°C/ $a_w$  0.75 and 45°C at all  $a_w$ .

The final moisture contents of the encapsulated powders of all 9 conditions were reported in Table 4, which varied from 6.5–12.8%. There was the influence of temperature, which can be observed in Table 4. At 35°C, the moisture content sharply increased through 5 weeks at all water activity levels whereas at 45°C the moisture content increased until week 3 and then slightly decreased. For 55°C, the moisture content increased highly in the first week but afterwards it gradually decreased. The change in moisture content might be due to the porous structure of encapsulated powder created by freeze drying. The samples firstly absorbed moisture when firstly exposed to high humidity. Then if the temperature was low, the moisture would continually absorb, but if high, the encapsulated powder might be dried, leading to a reduction of moisture content. Moreover, water activity was found not to have the influence on moisture content as shown in Table 4.

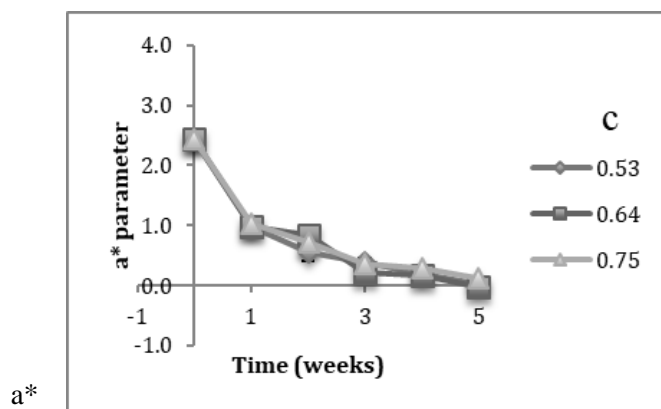
Based on all parameters, the temperature had negatively influenced on half-life, anthocyanin retention, and color quality of the encapsulated powder, whereas the water activity might cause some effects corresponding to the temperature. At 35°C and 45°C with low water activity, the conditions provided high half-life and anthocyanin retention; however, at 45°C the color was less changed over time. It was proposed that there were two mechanisms involved during 5-week storage. Firstly, the polymerization occurred as the evidence of decreased  $L^*$ , decreased  $b^*$ , and increased  $a^*$ . It might due to condensation of pigment with other phenolic compounds, the residual enzyme activity, and oxidation, which in turn changed the encapsulated powder's color. Anthocyanins in processed blueberries products and raspberry pulp were envisaged to polymerize during storage [18]. The second reaction might be the destruction of pigment due to heat, which converted anthocyanins to chalcone form. However, the compound generally would turn to be brown as claimed by Markakis [19], but obviously in this case, because of the presence of  $\beta$ -CD, the degraded anthocyanin possibly complexed with the cyclic oligosaccharide compound, decreasing the lightness. Moreover, the lower temperature was favored polymerization and when the storage time increased, the heat was accumulated and caused the anthocyanin hydrolysis. Besides, at high temperature, the evolvement of chalcone might occur faster so that the increasing



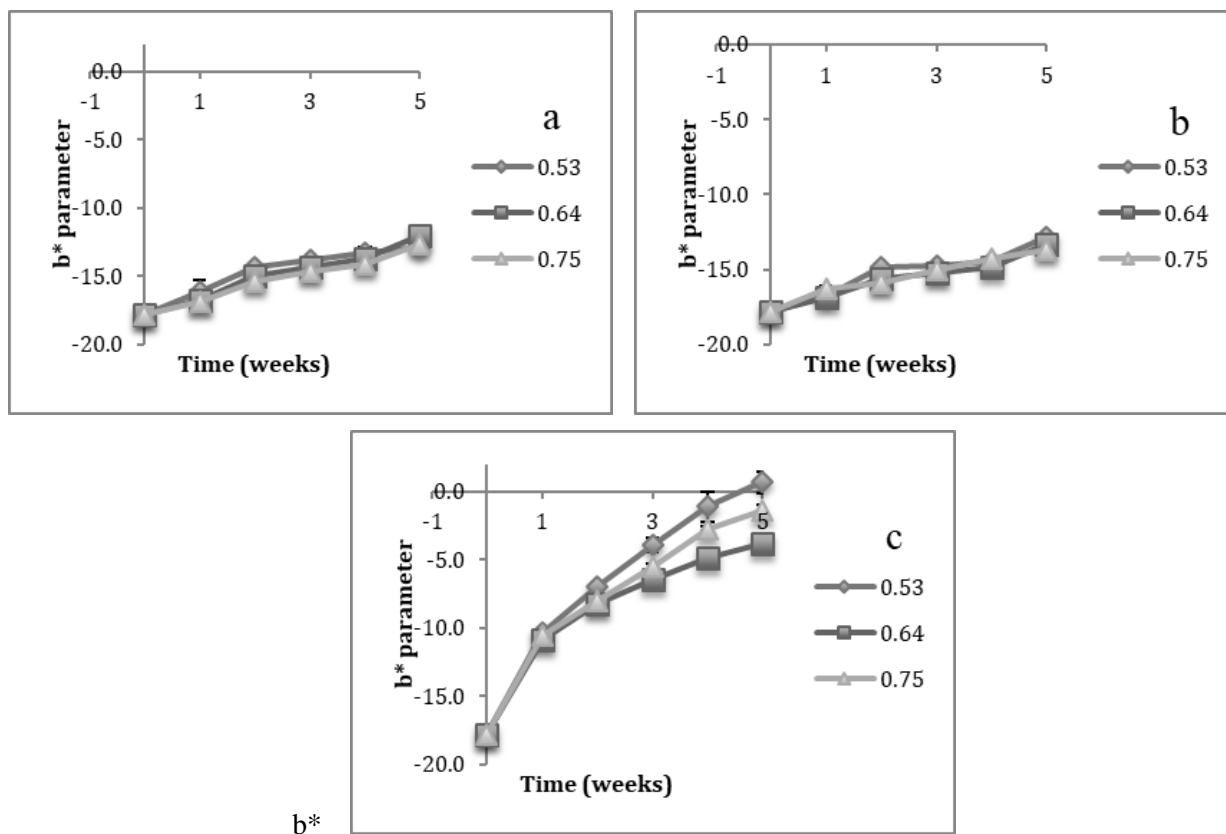
of lightness at 45°C underwent before those at 35°C. At 55°C the steady change of  $L^*$  was observed although  $a^*$  and  $b^*$  decreased rapidly.







**Figure 3:** The effect of temperature and water activity on  $L^*$  (Lightness),  $a^*$  (redness) and  $b^*$  (blueness) values during 5-week stability test, a) 35°C, b) 45°C and c) 55°C



**Figure 3 (continue):** The effect of temperature and water activity on  $L^*$  (Lightness),  $a^*$  (redness) and  $b^*$  (blueness) values during 5-week stability test, a) 35°C, b) 45°C and c) 55°C

## 4 CONCLUSIONS

Encapsulation of butterfly pea flower extract by using the ratio of the extract to the supporting materials (75% maltodextrin and 25%  $\beta$ -cyclodextrin) as 1:1 and freeze-drying method could retain the highest anthocyanin content as high as 88.3% with good color profile ( $L^* = 38.62$ ,  $a^* = 2.67$ , and  $b^* = -21.07$ ). An increasing of the supporting material proportion and  $\beta$ -cyclodextrin

tended to cause the color fading and reduced the anthocyanin content in the powder. The temperature and its interaction with water activity gave significant effect on the first order kinetic reaction of anthocyanin during the storage. The most suitable condition of storage was 45°C with  $a_w$  of 0.53 and 0.64, providing the kinetic constant ( $k$ ) of 0.1026 and 0.0881; and correlation coefficient ( $R^2$ ) of 0.9806 and 0.9906 with half-life of 6.75 and 7.88 weeks for  $a_w$  of 0.53 and 0.64, respectively.

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## PROCESSING OF BEVERAGES MADE FROM DANGSHEN ROOT (*CODONOPSIS PILOSULA*) BY-PRODUCTS AND WASTES

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### ABSTRACT

This study focused on the processing of the beverage from Dangshen root by-products and wastes to make full use of the raw materials. The study analyzed the quality criteria such as physico-chemical properties, bioactive compounds, nutritive values and microbiological characteristics to establish the basic standards for Dangshen root wastes for beverage processing. The Dangshen root waste (DRW): water ratio was 1:3 and the pectinase with concentration of 30 ppm was found to be effective in the extraction of phenolic compounds and antioxidants from Dangshen root wastes. In addition, the suitable extraction temperature and time were 100°C and 20 min, respectively to yield the highest total phenolic content ( $48.99 \pm 0.25$  mgGAE/100g DM) and antioxidant capacity ( $68.94 \pm 1.71^a$  mgTE/100g DM) of the Dangshen root waste beverages and ensure the safety for human consumption over 6 months of storage.

**Keywords:** Dangshen, waste, extraction. Antioxidant, beverage

### 1 INTRODUCTION

Dangshen scientifically known as *Condonopsis pilosula*, belongs to the Campanulaceae family. It has been used as a precious herb because dangshen contains many precious chemicals that are important for human health such as saponin, alkaloid, polyacetylene, inulin, protein, polyphenol and antioxidant [1, 2, 3, 4, 5, 7]. Especially saponin is an important ingredient for the production process of pharmaceuticals and creates the value of Dangshen.

Due to being grown on the ground, the harvesting of Dangshen using knives and scissors cannot avoid the mechanical damage resulting in a large portion (15-20%) of the damaged Dangshen that do not meet the standard of fresh trading. Moreover, some harvested Dangshen roots have unsatisfactory shape and quality such as curved, too long or too short shapes that cannot be sold in any market (5-10%). In addition, the Dangshen root part is considered by-products that also accounts for a significant amount (5%) in the postharvest stage. Thus, the amount of by-products and wastes not being used accounts for about 25-35% as stated by Cao Lam Co.Ltd).

Recently, there have been studies on the nutritional composition medicinal properties of Dangshen, however, the research on the Dangshen products is very limited, especially there are no studies on the use of Dangshen by-products and wastes in processing of food or beverages. Currently, the trend of consumers is to use non-carbonated beverage products, especially the herbs drink (green tea, herbal tea, pennywort, squash drink, etc.) with natural flavors. Consumption of these drinks has increased by 5-10% in recent years.

Therefore, the processing of beverages made from Dangshen root (*Codonopsis Pilosula*) by-products and wastes is needed to produce a highly valued product, minimize losses, increase economic efficiency in production and reduce environmental pollution.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Dangshen roots were harvested at Dangshen farm of Cao Lam Co., Ltd in Lam Dong province. Only those having physical damage were selected, washed and dried for the experiments. Honey, Stevia, Vitamin C, Potassium Sorbate were also used as additives and preservatives for making beverages.

### **2.2 Methods**

#### *2.2.1 Analysed Dangshen root waste materials*

The physico-chemical properties, bioactive compounds, nutritive values and microbiological characteristics of Dangshen root wastes (DRWs) were analyzed for making beverages.

#### *2.2.2 Established suitable parameters for making beverage from Dangshen root wastes*

##### ***Determined the suitable Dangshen root waste: water ratio***

The experiments were designed with 5 treatments at different ratios of fresh Dangshen root wastes to water from 1:2 to 1:6, the extraction temperature and the extraction time were fixed at 45°C and 60 minutes, respectively. The experiments were performed in triplicate

##### ***Investigated the effect of enzyme concentrations and extraction times on extract quality***

The Dangshen root waste: water ratio determined from the previous experiment was used in the enzyme extraction process by pectinase at the recommended temperature (45°C) from the supplier to obtain the highest extraction efficiency based on the quality of the extract. The experiment was randomly assigned in triplicate with experimental elements as enzyme concentrations (0-50ppm) and enzyme-assisted extraction time (1-3 hours).

##### ***Investigated pasteurization temperature and time to ensure the quality of products***

The experiments were designed with 5 treatments with pasteurization temperature of 85 to 100°C with an interval of 5°C for 5 to 20 minutes with an interval of 5 minutes. The experiments were performed in triplicate.

***Investigated the stability and shelf-life of Dangshen root waste beverages by accelerated aging method***

The DRW beverage samples were stored at  $50 \pm 2^\circ\text{C}$ ,  $75 \pm 5\%$  RH. The experiment designed with a randomized factor was conducted in 4 weeks. The method to determine shelf-life of products is to speed up the deterioration and degradation by the thermal acceleration (or Q method). The Q method assumes that the product quality degrades by a constant  $Q_n$  when the temperature changes to a certain number. With the temperature change interval is  $10^\circ\text{C}$ ,  $Q_n$  is called  $Q_{10}$ . For a known  $Q_{10}$  value, the expiry date can be calculated using the formula:

$$t_s = t_0 \cdot Q_{10}^n$$

Where:

$t_s$ : shelf-life at normal storage conditions.

$t_0$ : shelf-life estimation from accelerated storage condition

$n$ : (accelerated temperature  $^\circ\text{C}$  - normal storage temperature  $^\circ\text{C}$ )/ $10^\circ\text{C}$ .

***2.2.3 Methods of quality analysis and evaluation***

- Total soluble solids (TSS) were measured by ATAGO RX-5000 refractometer.
- Moisture content of Dangshen root wastes was measured by using Shimadzu-unibloc-electronic-moisture-balance-MOC12H
- Determination of the reducing sugar is based on the color reduction reaction between the reducing sugar and the DNS (3,5 - dinitrosalicylic acid) reagent.
- Total phenolic content (TPC) of dragon fruits was measured by using the Folin-Ciocalteu method as described by Singleton (1999) with some minor modifications.
- The DPPH radical scavenging activity was determined according to the method of Blois (1958) with modifications described by Brand-Williams et al. (1995), Fan et al. (2009) and Wojdylo et al. (2009).
- The quantification of saponins in DRWs is usually carried out by spectrophotometric methods [6].

**2.3 Data Analysis**

Data processing, comparison of experimental results were performed by using ANOVA method and identification of correlations between quality characteristics were done using MiniTAB 16 software.

### 3 RESULTS AND DISCUSSION

#### 3.1 Analysed Dangshen root waste materials

The physico-chemical properties, bioactive compounds, nutritive values and microbiological characteristics of Dangshen root wastes were analyzed for making beverages.

**Table 1:** The physico-chemical properties, bioactive compounds and nutritive values of fresh Dangshen root wastes

No.	Parameters	Units	Results
1	Moisture content	%	86.34 ± 2.36
2	Total soluble solid	°Brix	10,43 ± 1.04
3	Reducing sugar content	mg/g	1,13 ± 0,12
4	Total sugar content	%	22,31 ± 0,11
5	Total phenolic content	g/100ml	106,35 ± 2,16
6	Antioxidant capacity	μmolTE/g	181,63 ± 0,26
7	Saponin	mg/g	64,48 ± 1,58

**Table 2:** Microbiological characteristics of fresh Dangshen root wastes

No.	Parameters	Results	Methods
1	Total number of aerobic bacteria, CFU/g	1,4.103	TCVN 4884:2005
2	<i>Clostridium perfringens</i> , CFU/g	Not detected	TCVN 4991:2005
3	<i>Coliform</i> , MPN/g	2,3.103	TCVN 4882:2007
4	Supposed <i>E.coli</i> , MPN/g	Not detected	TCVN 6846:2007
5	<i>Staphylococcus aureus</i> , MPN/g	Not detected	TCVN 4830-3:2005
6	<i>Salmonella</i> (Qualitative in 25g)	(-)	TCVN 4829:2005

(-) Negative: There are no such bacteria in the sample

Note: The results of microbiological analysis from "Center for Analysis and Certification of Quality - Lam Dong Department of Science and Technology".

## 3.2 Established suitable parameters for making beverage from Dangshen root wastes

### 3.2.1 Determined the suitable Dangshen root waste: water ratio

**Table 3:** Effects of DRW: water ratio on the quality of Dangshen extract (n = 3)

DRW:water ratio	Dry matter content (%)	Brix (%)	Reducing sugar content (mg/100g dry matter)	Phenolic content (mgGAE /100g DM)	Antioxidant capacity (μmol TE/g DM)
1:2	2,01 <sup>a</sup> ±0,11	2,51 <sup>a</sup> ±0,21	405,46 <sup>b</sup> ±1,96	56,35 <sup>a</sup> ±1,13	70,00 <sup>a</sup> ±4,93
1:3	2,81 <sup>b</sup> ±0,38	2,81 <sup>b</sup> ±0,18	466,18 <sup>a</sup> ±2,46	66,13 <sup>b</sup> ±5,89	85,23 <sup>bc</sup> ±1,04
1:4	3,05 <sup>b</sup> ±0,21	3,05 <sup>b</sup> ±0,31	387,78 <sup>c</sup> ±1,32	77,16 <sup>b</sup> ±6,76	63,64 <sup>a</sup> ±5,14
1:5	3,35 <sup>b</sup> ±0,30	3,51 <sup>b</sup> ±0,20	251,40 <sup>d</sup> ±3,28	65,55 <sup>b</sup> ±5,73	81,38 <sup>bc</sup> ±4,60
1:6	3,11 <sup>b</sup> ±0,13	3,41 <sup>b</sup> ±0,13	243,46 <sup>e</sup> ±2,94	77,26 <sup>b</sup> ±7,32	91,38 <sup>c</sup> ±7,71

Different letters (a-d) within the same column show significant differences at  $p < 0.05$ . DM: Dry matter.

Table 3 shows that the reducing sugar content and antioxidant capacity of DRWs extracted with water at the ratio of 1:3 were highest among the sample extracts at different extraction ratios.

The ratios of 1:4, 1:5 and 1:6 reduced the extract content as larger volumes of water required longer time to heat the mixture to certain temperature (45°C) during the extraction period. There were no significant ( $p < 0.05$ ) differences in total phenolic content, dry matter and TSS of DRWs extracted with water at different ratios.

### 3.2.2 Investigated the effect of enzyme concentrations and extraction times on extract quality

**Table 4:** Effects of enzyme concentrations and extraction times on TPC of DRW extracts (n = 3)

Time	TPC (mg GAE/100g DM)					
	0ppm	10ppm	20ppm	30ppm	40ppm	50ppm
1h	67.50±0.38 <sup>a-v</sup>	69.98±0.25 <sup>a-v</sup>	93.97±1.63 <sup>ab-w</sup>	97.33±1.38 <sup>a-x</sup>	84.72±1.94 <sup>a-y</sup>	78.12±0.75 <sup>a-z</sup>
2h	84.72±0.56 <sup>a-w</sup>	85.78±0.56 <sup>b-w</sup>	92.73±0.63 <sup>a-x</sup>	93.66±0.31 <sup>b-x</sup>	87.15±0.50 <sup>a-y</sup>	83.12±0.56 <sup>b-z</sup>
3h	86.04±0.69 <sup>b-v</sup>	89.19±0.63 <sup>c-w</sup>	96.00±0.25 <sup>b-x</sup>	98.12±0.25 <sup>a-y</sup>	92.73±0.50 <sup>b-z</sup>	87.73±0.56 <sup>c-vw</sup>

Different letters (a-c) within the same column and (x-w) within the same row show significant differences at  $p < 0.05$ . DM: Dry matter.



**Table 5:** Effects of enzyme concentrations and extraction times on antioxidant capacity of DRW extracts (n = 3)

Time	Antioxidant capacity (μmol TE/g DM)					
	0ppm	10ppm	20ppm	30ppm	40ppm	50ppm
1h	73.64±0.64 <sup>a-x</sup>	77.27±3.21 <sup>a-xz</sup>	84.55±3.21 <sup>a-y</sup>	88.18±1.93 <sup>a-y</sup>	83.64±3.21 <sup>a-yz</sup>	82.27±2.57 <sup>a-yz</sup>
2h	60.00±3.21 <sup>b-x</sup>	77.27±3.21 <sup>a-z</sup>	86.82±2.57 <sup>a-y</sup>	87.73±1.29 <sup>a-y</sup>	79.55±2.57 <sup>a-z</sup>	73.18±2.57 <sup>a-z</sup>
3h	52.73±1.93 <sup>c-x</sup>	55.91±1.29 <sup>b-xy</sup>	61.36±2.57 <sup>b-yz</sup>	66.36±1.93 <sup>b-z</sup>	55.91±2.57 <sup>b-xy</sup>	54.09±5.14 <sup>b-x</sup>

*Different letters (a-c) within the same column and (x-z) within the same row show significant differences at  $p < 0.05$ . DM: Dry mater.*

The interaction effects of the two factors including enzyme concentration and extraction time were found non-significant (Table 4 and Table 5). The pectinase concentration greatly affected the increase in TPC and antioxidant capacity of DRW extracts while effect of extraction time was less.

There were no statistically significant differences between the antioxidant capacity of DRWs extracted by pectinase at concentrations of 20 ppm, 30 ppm, and 40 ppm. However, the samples extracted by 30 ppm of pectinase showed higher antioxidant capacity than those treated by 10 ppm and 50 ppm of pectinase. Table 4 showed that TPC increased as the concentration of pectinase in the range from 10-30 ppm increased. The concentrations of pectinase from 40 to 50 ppm decreased the TPC of DRW extracts.

Enzymatic assisted extraction of TPC and antioxidants from the DRWs may occur via hydrolytic degradation of the cell wall polysaccharides, which can retain phenolics in the polysaccharide-lignin network by hydrogen or hydrophobic bonding. Moreover, the direct enzyme catalyzed breakage of the ether and/or ester linkages between the phenols and the plant cell wall polymers [8]. When substrate is excessive, the reaction velocity increases as the enzyme level increases but when the enzyme concentration is saturated in the substrate, the reaction velocity does not change or could be decrease when the enzyme concentration increases.

The results of Table 4 and Table 5 also showed that the extraction time did not significantly affected total phenolic content and antioxidant capacity of DRW extracts. In theory, the longer the extraction time, the greater the amount of compounds since it will increase the contact time between the material and the solvent, thus increasing the diffusion of molecules from the material into the solution and the extraction performance. However, excessive extraction time will not be effective. During the extraction process, the total phenolic content and antioxidant capacity of DRW extracted in different extraction times were similar since the concentration of the extracted substances in the DRWs and in the solution reaches equilibrium so the longer extraction time will not increase the amount of extract.

**Table 6:** Effects of enzyme concentrations and extraction times on reducing sugar content of DRW extracts (n = 3)

Time	Reducing sugar content (mg/100g DM)					
	0ppm	10ppm	20ppm	30ppm	40ppm	50ppm
1h	449.26±1.75 <sup>a-x</sup>	460.37±0.87 <sup>a-y</sup>	463.46±0.87 <sup>a-y</sup>	468.70±2.18 <sup>a-z</sup>	463.15±1.31 <sup>a-y</sup>	461.91±1.31 <sup>a-y</sup>
2h	479.81±0.44 <sup>b-x</sup>	484.14±0.44 <sup>b-y</sup>	490.00±1.75 <sup>b-z</sup>	492.47±0.87 <sup>b-z</sup>	471.17±0.44 <sup>b-w</sup>	471.79±0.44 <sup>b-w</sup>
3h	265.93±3.49 <sup>c-x</sup>	277.96±2.18 <sup>c-y</sup>	287.22±1.31 <sup>c-y</sup>	372.72±1.75 <sup>c-z</sup>	230.43±3.06 <sup>c-w</sup>	228.27±4.36 <sup>c-w</sup>

Different letters (a-c) within the same column and (x-w) within the same row show significant differences at  $p < 0.05$ . DM: Dry mater.

Table 6 showed that there was no significant difference in TSS between DRWs extracted with and without enzyme assisted.

### 3.2.3 Effects of pasteurization temperature and time on the quality of the products

**Table 7:** Effects of pasturization temperature and time on the total phenolic content of DRW extracts (n = 3)

Temp	TPC (mg GAE/100g DM)			
	5 min	10 min	15 min	20 min
85°C	40.32±0.50 <sup>a-x</sup>	41.12±0.13 <sup>a-xy</sup>	41.65±0.13 <sup>a-yz</sup>	42.27±0.25 <sup>a-z</sup>
90°C	41.47±0.13 <sup>b-x</sup>	42.00±0.38 <sup>b-x</sup>	42.44±0.25 <sup>a-xy</sup>	43.33±0.75 <sup>a-xy</sup>
95°C	47.75±0.25 <sup>c-x</sup>	49.17±0.25 <sup>c-y</sup>	50.41±0.75 <sup>b-z</sup>	50.67±0.13 <sup>b-z</sup>
100°C	48.37±0.013 <sup>x</sup>	48.64±0.25 <sup>c-x</sup>	48.81±0.25 <sup>c-x</sup>	48.99±0.25 <sup>c-x</sup>

Different letters (a-c) within the same column and (x-z) within the same row show significant differences at  $p < 0.05$ . DM: Dry mater.

**Table 8:** Effects of pasturization temperature and time on the antioxidant capacity of DRW extracts (n = 3)

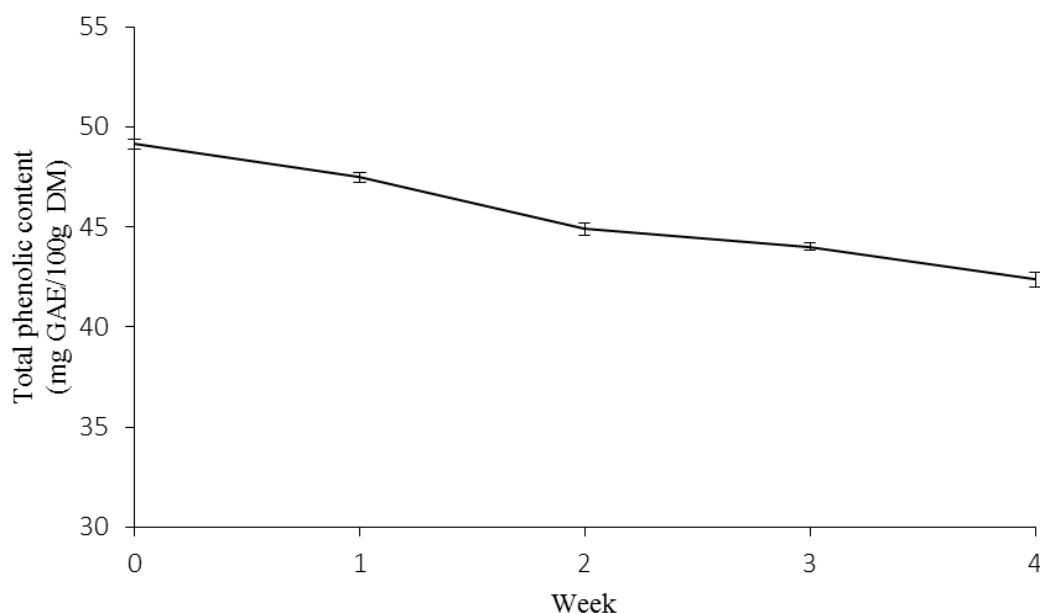
Temp	Antioxidant capacity (μmol TE/g DM)			
	5 min	10 min	15 min	20 min
85°C	55.45±1.50 <sup>a-x</sup>	62.73±0.64 <sup>a-y</sup>	63.18±0.00 <sup>b-y</sup>	65.91±2.14 <sup>ab-y</sup>
90°C	57.12±0.86 <sup>a-x</sup>	60.00±0.21 <sup>b-y</sup>	60.76±0.43 <sup>c-y</sup>	63.79±0.86 <sup>bc-z</sup>
95°C	53.03±1.07 <sup>a-x</sup>	57.58±0.64 <sup>bc-y</sup>	59.39±0.21 <sup>d-y</sup>	60.15±0.43 <sup>c-y</sup>
100°C	56.36±1.93 <sup>a-x</sup>	59.09±0.64 <sup>c-x</sup>	65.15±0.64 <sup>a-y</sup>	68.94±1.71 <sup>a-y</sup>

Different letters (a-c) within the same column and (x-z) within the same row show significant differences at  $p < 0.05$ . DM: Dry mater.

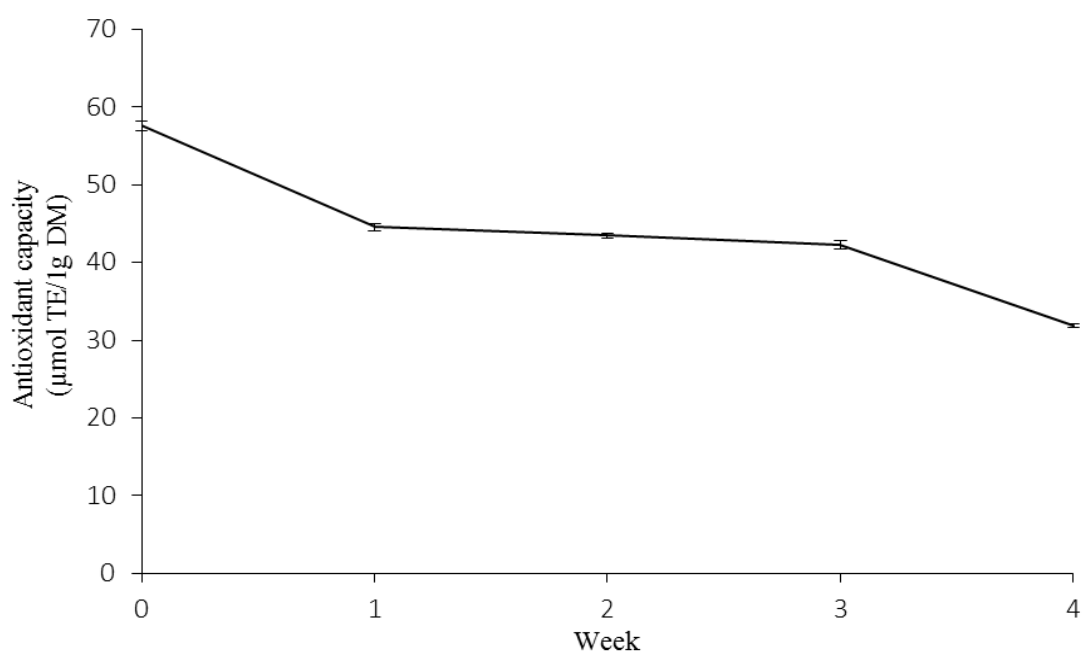
Table 7 and Table 8 show that pasteurization temperature had no significant effects on the quality of DRWs, whilst at the same temperature, the pasteurization time had a significant effect on total phenolic content and antioxidant capacity ( $p < 0.05$ ). DRW extracts pasteurized at 20 min showed high total phenol content and antioxidant capacity, especially those pasteurized at 100°C gave the highest values.

Most published studies on the effects of thermal treatment on the total phenolic content and antioxidants show the contradicting results. Some researchers reported an increase in the phenolic content and antioxidant capacity whilst others observed a decrease. Most losses are due to the degradation of phenolic and antioxidant compounds by heating. Simultaneously, leaching of these compounds from the Dangshen roots into the water during the prolonged exposure to water and heat may give benefits as the antioxidant compounds can be retained in the extracts or beverages. Seok et al (2004)[9] also suggested that phenolic compounds in plant cells can be liberated by heat treatment, therefore, these compounds increase as the treatment temperature increases.

#### 3.2.4 Investigated the stability and shelf-life of Dangshen root waste beverages by accelerated aging method



**Figure 1:** The total phenolic content (mg GAE/100g DM) of Dangshen root waste beverages over 4 weeks of storage in accelerated conditions



**Figure 2:** The antioxidant capacity Antioxidant capacity (μmol TE/1g DM) of Dangshen root waste beverages over 4 weeks of storage in accelerated conditions

The quality and microbiological characteristics of DRS beverages were monitored over 4 weeks of storage in the accelerated conditions (50°C, 75% RH). Although the TPC and antioxidant capacity of DRW beverages significantly ( $p < 0.05$ ) decreased after four weeks, the values of products were high. The quality of DRW beverages after four weeks in accelerated storage conditions was compatible with those stored after 6 months in the normal condition (30°C, 75% RH).

**Table 9:** Microbiological quality of DRW extracts

Week	Microbiological Quality (CFU/ml)		
	Total Plate Count (TPC)	Mold Count (MC)	Yeast Count (YC)
Week 0	0.33±0.58	0.00±0.00	0.00±0.00
Week 1	0.33±0.58	0.33±0.58	0.00±0.00
Week 2	0.33±0.58	0.00±0.00	0.00±0.00
Week 3	0.33±0.58	0.00±0.00	0.00±0.00
Week 4	0.33±0.58	0.33±0.58	0.00±0.00
Satisfactory	≤ 100	≤ 10	≤ 10

\*The National Technical Standard for Non-Alcoholic Beverages (QCVN 6-2: 2010 / BYT)

In addition, the microbiological characteristics such as the total plate count (TPC), molds and yeasts of DRW beverages over 6 months of storage (equivalent to 4 weeks in accelerated storage conditions) were within acceptable limits according to The National Technical Standard for Non-Alcoholic Beverages (QCVN 6-2: 2010 / BYT).

#### **4.CONCLUSIONS**

The study determined optimum processing conditions and assessed quality to develop the Dangshen root waste beverages that contain high total phenolic content and antioxidant capacity. The Dangshen root wastes to water ratio was 1:3 and the pectinase with concentration of 30 ppm was found to be effective in the extraction of phenolic compounds and antioxidants from Dangshen root wastes. In addition, the suitable extraction temperature and time were 100°C and 20 min, respectively to yield the highest total phenolic content and antioxidant capacity of the Dangshen root waste beverages and ensure the safety for human consumption over 6 months of storage.

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## STUDY OF ULTRASOUND-ASSISTED EXTRACTION OF PHENOLIC COMPOUNDS FROM XAO TAM PHAN (*PARAMIGNYA TRIMERA*)

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### ABSTRACT

An efficiency of ultrasound-assisted extraction (UAE) of XaoTamPhan (*Paramignya trimera*) bioactive compounds (XBCs) was established and antioxidant activity of XBCs was studied. In this study, XBCs were extracted by UAE using water as extraction solvent. The UAE technological parameters such as material:solvent ratio, ultrasonic power and treatment time were investigated. The results showed that an extraction at 1:20 g dry weight (DW)/mL, 150 W/g DW for 12 mins was the most effective for UAE of XBCs. On the basis of second-order kinetic model of extraction process, the extraction rate constant of total phenolics in UAE increased approximately 2.3 times in comparison with that in conventional extraction. In addition, the maximal level of total phenolics and the antioxidant activity evaluated by DPPH and FRAP methods in UAE were 9.59 mg GAE/g DW, 11.12 mg TE/ g DW and 12.92 mg TE/ g DW, respectively. Thus, UAE is recommended for the improvement of bioactive compounds and increase the antioxidant activity of the extract from Xao Tam Phan (*Paramignya trimera*).

**Keywords:** Antioxidant activity, extraction kinetics, *Paramignya trimera*, ultrasound-assisted.

### 1 INTRODUCTION

Xao tam phan (*Paramignya trimera* Oliv. Burkill) is a woody shrub, mostly distributed in the Southern regions of Vietnam and used as a medicinal plant to treat liver diseases and cancer. Recent studies on the *P. trimera* have revealed hepatoprotective and cytotoxic activity from a crude methanolic extract [5; 12]. Phenolics and ostruthin were major compounds in the *P. trimera* roots and stems, and displayed potent activity against pancreatic cancer cell lines [9]. *P. trimera* contains key biologically active phytochemicals and it is imperative to optimize extraction conditions to maximize the yield of phytochemicals extracted for potential medicinal exploitation.

Solvent is one of the direct factors to extraction efficiency and relates to production costs [2]. One of the solvents commonly used to extract polyphenol from Xao tam phan *P. trimera* is methanol [3], but this solvent is toxic and usually restricted to use in food production [1]. In recent years, advanced extraction techniques have been developed and applied to optimize the

extraction of phenolics from plant materials. According to some authors, water is unsuitable solvent used for extracting bioactive compounds from Xao tam phan [3]. However, ultrasonic treatment can strongly support the extraction process with water solvent to produce food extracts [6].

In particular, ultrasound-assisted extraction (UAE) not only increases the content of antioxidants in the extract but also reduces the extraction time, reduces the amount of solvent needed and has high automation ability than other extraction techniques [6]. Recently, the results of the use of ultrasound to support the extraction of antioxidant compounds from Xao tam phan are still limited.

The determination of kinetic parameters should be very important for designing efficient ultrasound-assisted extraction process for antioxidant production from Xao Tam Phan. Nevertheless, no relevant kinetic model of ultrasound-assisted antioxidant extraction was reported.

The objectives of this research were to (1) study the effects of technological factors of ultrasound-assisted extraction on total phenolic content, antioxidant activity and (2) determine the kinetic parameters that describe the mechanism of ultrasound-assisted extraction.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

*P. trimera* root was collected from Ninh Thuan province. It was cut into pieces and then the pieces was washed and dried, stored at room temperature. Subsequently, the material was crushed into fine powder (0.2mm) and was used for all experiments.

### **2.2 Chemicals**

All chemicals used were of analytical grade. 1,1-Diphenyl-2-picryl-hydrazil (DPPH), Folin-Ciocalteu, 2,4,6-tripyridyl-s-triazine (TPTZ), iron (III) chloride and standard compounds including gallic acid, trolox were purchased from Sigma–Aldrich Pty Ltd.

### **2.3 Extraction performance by ultrasound**

Xao tam phan powder (3g) was immersed into 60 mL of distilled water. The extraction process was performed using an ultrasonic probe (VCX 750, 20 kHz, Sonics & Materials Inc., USA) with different material:solvent ratio (1:10, 1:20, 1:30, 1:40 and 1:50 mg DW/mL solvent), ultrasonic power (50, 100, 150, 200 and 250 W/g DW ) and treatment time (0 to 10 min). For all experiments, the control samples were untreated with ultrasound. At the end of the process, the extract was centrifuged at 12.000 rpm for 5 min and supernatant was collected to determine total phenolics and antioxidant activity.



## 2.4 Determination of kinetic parameters of phenolic extraction from the second-order kinetic model

To determine the extraction rate constant of total phenolics, the second-order rate law was applied [14]. The general second-order model can be written as:

$$\frac{dC_t}{dt} = k(C_e - C_t)^2 \quad (1)$$

where,  $k$  is the second-order extraction rate constant ( $\text{Lg}^{-1} \text{min}^{-1}$ ),  $C_e$  is the extraction capacity (the equilibrium concentration of total phenolics and the antioxidant activity evaluated by DPPH in Xao Tam Phan extract ( $\text{gL}^{-1}$ ), and  $C_t$  is the concentration of total phenolics in Xao Tam Phan extract at a given treatment time ( $\text{gL}^{-1}$ ). The integrated rate law for a second-order extraction, under the boundary conditions  $t = 0$  to  $t$  and  $C_t = 0$  to  $C_t$ , can be written as an equation (2) or a linearized equation (3):

$$C_t = \frac{C_e^2 kt}{1 + C_e kt} \quad (2)$$

$$\frac{1}{C_t} = \frac{1}{kC_e^2} + \frac{1}{C_e} \quad (3)$$

The initial extraction rate,  $h$  ( $\text{gL}^{-1} \text{min}^{-1}$ ), when  $t$  approaches 0, can be defined as equation (4)

$$h = hC_e^2 \quad (4)$$

After rearranging the Eqs. (3) and (4),  $C_t$  can be expressed as:

$$C_t = \frac{1}{\frac{1}{h} + \frac{1}{C_e}} \quad (5)$$

The initial extraction rate,  $h$ , the extraction capacity,  $C_e$ , and the second-order extraction rate constant,  $k$ , can be determined experimentally from the slope and the intercept by plotting  $t/C_t$  against  $t$ .

## 2.5 Analytical methods

### *Total phenolic content (TPC)*

TPC of *P. trimera* extract was determined using the Folin–Ciocalteu method as previously described by Vuong et al. [15] with some modifications. Briefly, 0.125 mL of the extracts were mixed with 0.125 mL Folin–Ciocalteu reagent and 1.5 mL distilled water. The mixture was left to settle for 6 min, then 1.25 mL of 7.5% (w/v)  $\text{Na}_2\text{CO}_3$  solution was added and incubated in the

dark at room temperature for 90 min. The absorbance of mixture was measured at 765 nm using a UV–vis spectrophotometer. Distilled water and gallic acid were used as a control and standard. TPC was expressed in mg gallic acid equivalents (GAE)/g DW.

#### *DPPH radical scavenging capacity*

DPPH radical scavenging capacity of *P. trimera* extract was measured based on the method reported by Vuong et al. [15] with some modifications. Briefly, a stock solution of 0.024% (w/v) DPPH (1,1-diphenyl-2-picryl-hydrazil) in methanol was prepared and stored at  $-18^{\circ}\text{C}$ . Before use, a working solution was prepared by diluting 1.0 mL of stock solution with 45 mL of methanol to obtain an absorbance of  $1.1 \pm 0.02$  at 515 nm. For the DPPH reaction, 0.15 mL of the extract was mixed with 2.850 mL of the working solution and incubated for 30 min in the dark at room temperature. The absorbance of mixture was measured at 515 nm using a UV–vis spectrophotometer. The results were expressed as mg trolox equivalents (TE)/g DW.

#### *Ferric-reducing antioxidant power*

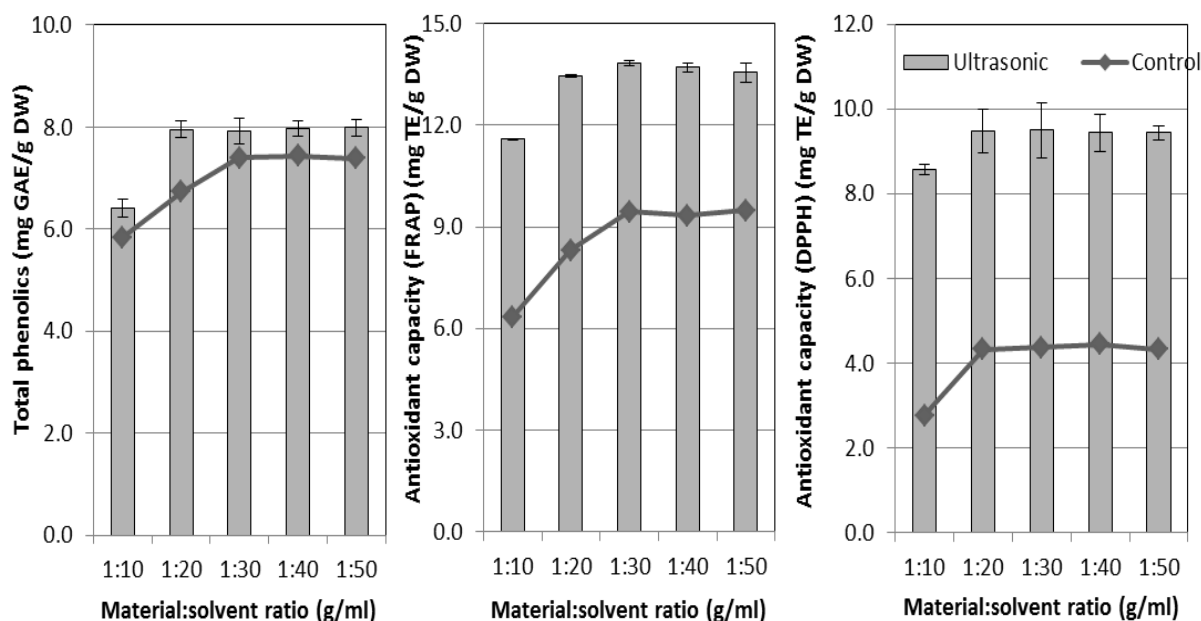
The ferric-reducing antioxidant power (FRAP) of the *P. trimera* extract was determined according to the method as reported by Nguyen et al [13]. Reagents including reagent A: 300 mM acetate buffer solution, pH 3.6; reagent B: 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl; and reagent C: 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution. Before use, the fresh FRAP solution was prepared by mixing reagents A, B, and C at a ratio of 10:1:1. For the FRAP reaction, 0.15 mL of the extract was mixed with 2.850 mL of the fresh FRAP solution and incubated in the dark at room temperature for 30 min. The absorbance of the mixture was read at 593 nm using a UV-Vis spectrophotometer. Methanol and Trolox were used as a control and standard, respectively. The results expressed as milligrams of Trolox equivalents (TE)/g DW.

## **2.6 Statistical analysis**

All experiments were performed in triplicate. The experimental results obtained were expressed as means  $\pm$  SD (standard deviation). Mean values were considered significantly different when  $P < 0.05$ . Analysis of variance was performed using the software Statgraphics plus, version 7.0 (Manugistics, Inc., Rockville, MD, USA).

### 3 RESULTS AND DISCUSSION

#### 3.1 Influence of extraction parameters



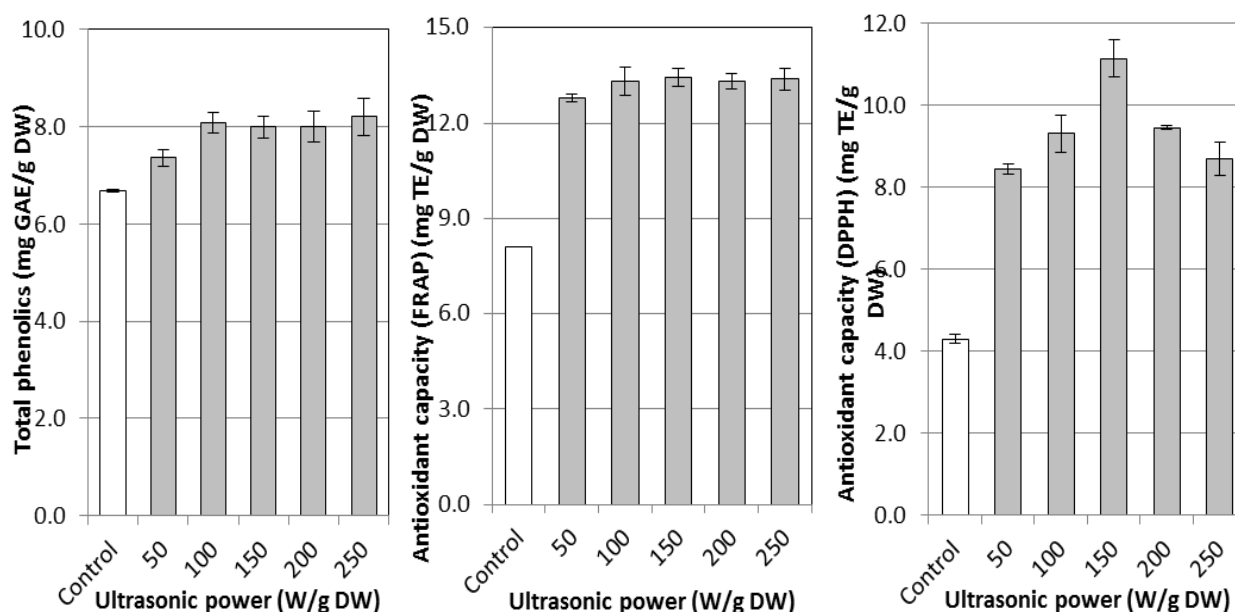
**Figure 1:** Effect of material:solvent ratio on total phenolics and antioxidant activity

##### 3.1.1 Effect of material:solvent ratio

In this study, distilled water was chosen as solvent because it is suitable for use in food production. In addition, ultrasonic treatment was also used to support extraction process. The total phenolics and antioxidant activity from *P. trimera* extract with different material:solvent ratio are shown in Figure 1.

It can be seen that the total phenolic content increased rapidly when the material:solvent ratio decreased from 1:10 to 1:20 g DW/ml solvent and then reached the final stabilization. Meanwhile the sample without ultrasonic treatment got stable at 1:30 g DW/ml solvent. The antioxidant capacity evaluated by DPPH and FRAP methods increased 10.57% and 16.07% when the material:solvent ratio decreased from 1:10 to 1:20 (g DW/ml solvent) and these values were 2.19 and 1.62 times higher than that of the control sample, respectively. However, further decrease material:solvent ratio from 1:30 to 1:50 did not significantly increase the antioxidant capacity ( $P > 0.05$ ). In general, lower material:solvent ratio resulted in a larger concentration gradient during the diffusion from internal material into the solution, leading to the increase of antioxidant compounds and activity. Similarly, Li et al [10] also described that the extraction capacity of chlorogenic acid increased with the decrease of the sample:solvent ratio from *Eucommia ulmoides*.

### 3.1.2 Effect of ultrasonic power



**Figure 2:** Effect of ultrasonic power on total phenolics and antioxidant activity

Figure 2 illustrates the effect of ultrasonic power on the level of total phenolic compounds and antioxidant activity of Xiao Tam Phan extract. The results showed that, when the ultrasonic power increased to 100 W/g DW, the total phenolic content and antioxidant activity evaluated by FRAP increased 1.21 and 1.64 times compared to the sample without ultrasonic treatment, respectively. The antioxidant activity of the extract evaluated by FRAP is strongly related to total phenolics of Xiao Tam Phan. These values reached maximum at the same ultrasonic power and kept unchanged when the ultrasonic power was higher than 100 W/g DW. A similar study reported that ultrasonic powers from 3.2 to 56 W significantly increased the yield of extracted phenolic compounds from *Satsuma Mandarin* peels by 58–82% [11].

Unlike antioxidant activity evaluated by FRAP, the antioxidant activity evaluated by DPPH was highest at 150 W/g DW by  $11.13 \pm 0.45$  mg TE/g DW. A further increase in the ultrasonic power resulted in significantly ( $P < 0.05$ ) lower antioxidant activity evaluated by DPPH.

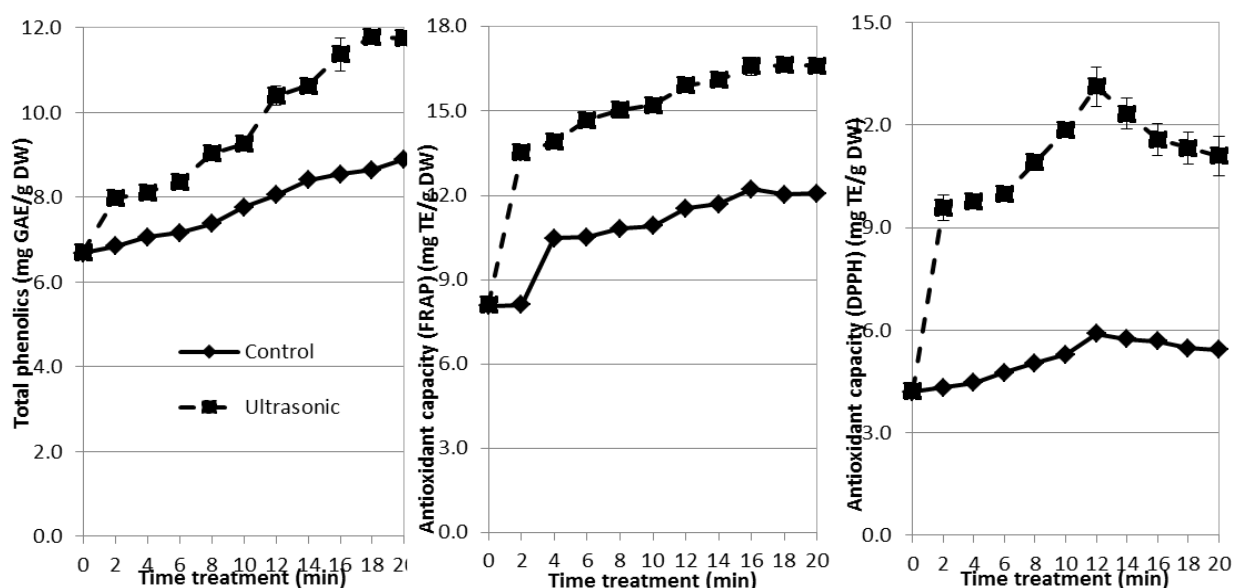
It is believed that the increase in antioxidant compounds and antioxidant activity was mainly due to the improved cavitation and mechanical effect of ultrasound which increased the contact surface area between solid and liquid phases and caused greater penetration of solvent into the material matrix. Simultaneously, the ultrasonic process in the liquid will produce free radicals and oxidizing components in raw materials [4].

In previous study, the phytochemical compounds of Xiao Tam Phan was a rich source of saponins. Beside phenolic compounds, ostruthin was a major compound identified in the roots and stems of the *P. trimera* [13] and also contributed to the antioxidant activity evaluated by DPPH of the extract. The increase of ultrasonic power (higher than 150 W/g DW) probably

caused the oxidation of bioactive compounds such as ostruthin leading to the decrease of antioxidant activity evaluated by DPPH.

### 3.1.3 Effect of treatment time

The effect of treatment time on the total phenolic content and antioxidant activity evaluated by FRAP and DPPH is shown in Figure 3. The results showed that the conventional extraction method was more time consuming for mass transfer than the ultrasonic-assisted extraction method. The total phenolic content and the antioxidant capacity evaluated by FRAP were stable at 18 and 16 min, and reached value of  $11.78 \pm 0.1$  mg GAE/g DW and  $16.59 \pm 0.31$  mg TE/g.



**Figure 3:** Effect of treatment time on total phenolics and antioxidant activity

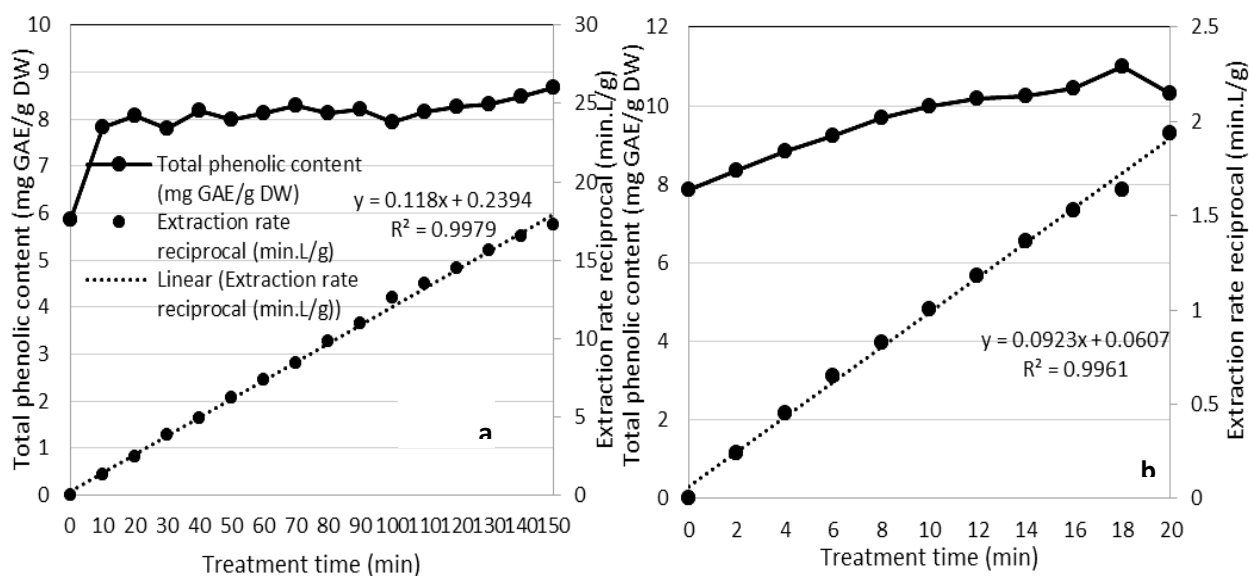
DW, respectively. In contrast, the antioxidant capacity evaluated by DPPH reached a maximum at 12 minutes corresponding to  $13.12 \pm 0.57$  mg TE/g DW.

The mechanism of UAE is involved to the acoustic cavitation, which includes the formation, growth and implosive collapse of bubbles in a liquid. The implosion of cavitation bubbles generates severe turbulence, high-velocity interparticle collisions and perturbation in microporous particles of the materials, which accelerates the eddy diffusion and internal diffusion. Moreover, cavitation within the proximity of solid surface causes surface erosion and particle breakdown [4].

A longer ultrasonic treatment time might present a negative effect on the antioxidant activity evaluated by DPPH. Long-term ultrasonic treatment did not increase the polyphenol content and antioxidant capacity of the extract, due to ultrasonic damage to the polyphenol structure of the extract during long-term extraction [7].

### 3.2 Kinetics of ultrasound-assisted extraction (UAE)

Figure 4 presents the linearized forms of the second-order model for the two different extraction methods. The kinetic parameters were determined from the slope and intercept by plotting  $t/C_t$  against  $t$  and listed in Table 1. The results indicated that  $k$ ,  $C_e$  and  $h$  in UAE were higher than that of without-ultrasonic sample. This verified that the ultrasound-assisted extraction could greatly improve the extraction rates of total phenolics from *P. trimera*. The second-order model fitted well the experimental results because of the obtained high coefficient of determination ( $R^2 = 0.9961$ – $0.9979$ ). For phenolic compound extraction process, the values of the rate constant ( $k$ ), the extraction capacity ( $C_e$ ) and the initial extraction rate ( $h$ ) of UAE were found higher approximately 2.3, 1.28 and 3.94 times than that of without-ultrasonic treatment, respectively. Similar kinetic effects were reported by Khan et al [8] for UAE of polyphenols from orange peel.



**Figure 4:** Extraction rate reciprocal ( $t/C_t$ ) of total phenolics from Xao Tam Phan extract at different extraction times ( $t$ ) for without-ultrasonic extraction (a) and ultrasonic-assisted extraction (b)

**Table 1:** The second-order kinetic parameters of ultrasonic-assisted extraction of phenolic compounds

Method	The initial extraction rate, $h$ ( $\text{g L}^{-1} \text{min}^{-1}$ ),	Total phenolic content - $C_e$ (mg GAE/g DW)	The second-order extraction rate constant- $k$ ( $\text{L g}^{-1} \text{min}^{-1}$ )	$R^2$
Without ultrasonic	4.18	8.47	0.06	0.9979
Ultrasonic-assisted	16.47	10.83	0.14	0.9961

## **4 CONCLUSIONS**

The study has indicated that the use of ultrasonic treatment improved extraction efficiency of total phenolics and increased antioxidant activity of Xao tam phan extract. Furthermore, a considerable reduction in the extraction time seems to be suitable for the extraction of thermally labile compounds. Because of low electrical energy consumption, high extraction time reduction, antioxidant compounds and antioxidant activity increase, UAE was clearly better than conventional extraction. A second-order kinetic model was successfully applied to describe the mechanism of ultrasound-assisted extraction of phenolic compounds from Xao Tam Phan.

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# SCREENING AND OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF BETACYANIN AND TOTAL PHENOLIC COMPOUNDS FROM DRAGON FRUIT PEEL (*HYLOCEREUS UNDANTUS*)

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## ABSTRACT

Dragon fruit is among valuable tropical fruits of Binh Thuan province in Vietnam. The flesh is the main material for processing of dried dragon fruit products or fermented dragon fruit juice and the peel is usually waste. However, the peel of dragon fruit contains betacyanin, the reddish color, which can be used as a natural food colorant. This study aimed to extract betacyanin from dragon fruit peel with the support of ultrasound. Five technological factors of ultrasound-assisted extraction process that affect betacyanin content in the extract were type of material, liquid solid ratio, extraction temperature, ultrasonic treatment time and ultrasound capacity. By using Plackett-Burman experimental design, three out of five technological factors were selected to further optimization. The response surface method (RSM) was used to determine optimum extraction parameters. At the optimum ultrasound-assisted extraction parameters with ultrasonic treatment time 3.82 min, ultrasound capacity 55.05 W/g<sub>dry weight</sub> and extraction temperature 53.4°C, the achieved betacyanin content in the extract was maximum and reached  $21.17 \pm 0.500$  mg/100g<sub>dry weight</sub>.

**Keywords:** Dragon fruit peel, betacyanin, ultrasound-assisted extraction, optimization.

## 1 INTRODUCTION

Recently food manufacturers use the synthesized colorants which potentially influence the health of consumers, so current research focuses on the exploitation of natural pigments from plants origins like flowers, bulbs, leaves, stems, woods, fruits, and seeds. These sources are safer and nontoxic to end user and become an important alternative to synthesized food colorants. In addition, there is a developing tendency to exploit food colorants from microbial, animals and minerals origins [1]. White flesh dragon fruit (*Hylocereus undantus*) belongs to the *Vine cacti* from the family of *Cactaceae* cultivated mainly in Southern East provinces of Vietnam such as Binh Thuan, Ninh Thuan and Southern West provinces like Long An, Tien Giang. According to

the report of People committee of Binh Thuan province, dragon fruit has been recognized as a specific fruit of Binh Thuan since 2011.

Due to the suitable climate of Binh Thuan for cultivation, the yield of dragon fruit of this area in 2015 reached 500,000 metric tons. Dragon fruits flesh is the main ingredients of dragon fruit juice, wine, snack but the peel has not been fully utilized. According to the research of Lou et al [2], the phytochemical bioactive compounds in two types of dragon fruit peel were extracted by supercritical carbon dioxide and analyzed by HPLC. The inhibition of cancer cell *in vitro* condition of the extract was studied and the results showed that the extract can inhibit the growth of cancer cells.

The red-purple pigments belong to a water-soluble nitrogen-containing pigment group called betalains. Betalains in dragon fruit consist of mainly betanin, isobetanin, phyllocactin I-II, isophyllocactin, descarboxy neobetainidin. These compounds belong to the betacyanin group with the reddish color, the yellow-orange betalains called betaxanthin group appear with trace amount in dragon fruit [3]. Betacyanin in dragon fruit peel is potential source of natural food colorant that can be used to substitute synthesized dyes. In the USA, the beetroot is the only allowed source for producing betalains (E-162) as food additives [4]. However, the other promising source of betalains such as dragon fruit peel can also play an important role in producing food colorants.

Several organic solvents like methanol, acetone or ethanol have been used to extract betacyanin [1, 5]. However the problem is organic solvent probably harm to human health, so distilled water was used as extraction solvent due to the harmlessness of water and low-cost. Betacyanin is highly sensitive to heat and other factors like high pH (>6), light, water activity, metal ions and air [6]. In previous studies, the material was submerged in solvent and extracted for long time. These shortcomings led to the development of using assistant techniques such as microwave, enzymatic treatment, ultrasound or combination of them.

In ultrasonic-assisted extraction method (UAE) method, the ratio between solvent and material, extraction temperature, ultrasound capacity and ultrasonic treatment time strongly affect the yield of extraction. The ultrasonic waves produce micro-bubble in the medium, these bubbles then burst and create shear-force or micro-jet effect, leading to cell wall breaking and increase in the absorption of solvent into the material, which helps the compounds inside move out easily [7, 8].

In this study, the extraction of betacyanin from dragon fruit peel was carried out by using ultrasonic-assisted extraction method (UAE) with distilled water as solvent. Technological factors of ultrasound-assisted extraction process that strongly affect betacyanin content in the extract were screened by using Plackett-Burman experimental design and then the response surface method (RSM) was used to determine optimum extraction parameters.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Ripen dragon fruits were selected and purchased from Lagi, Binh Thuan province. Fruits were washed with tap water, the peels were separated from flesh and blanched with hot steam at 90-95°C for 3 minutes [9]. The peels were vacuum-sealed and frozen at -10°C for further use. All the chemicals used in this study were analytical grade.

### 2.2 Extraction of pigment

Ultrasound-assisted extraction was carried out by using ultrasound processors VCX 750 (SONICS, USA) with flat tip 1.0 cm probe transducer. The frozen peels were ground with blender (Panasonic, Malaysia) then mixed with distilled water. The obtained mixture was adjusted pH to 5.5, ultrasonic treated and then incubated in the water bath for betacyanin extraction (Memmert, Germany). For each trial, 6g of peel mash were placed into 250ml beakers, after extraction the mixture was filtered by cheese cloth to separate the residue and filtered again with filter paper to obtain the clear filtrate. In this study, extraction parameters were investigated as follow: ultrasonic time from 3 to 6 minutes, temperature of extraction process from 50°C to 70°C, liquid solid (LS) ratio (mL of solvent per g of material) from 10 : 1 to 30 : 1, ultrasound capacity from 25W/g<sub>dry weight</sub> to 75W/g<sub>dry weight</sub>.

### 2.3 Determination of total betacyanin content

The betacyanin content was determined by spectrophotometric method based on the previous researches of Bakar et al [10] and Cai et al [11]. The betacyanin content was calculated from spectrophotometric measurements at absorption wavelength 538 – 540nm. The following equation was applied to determine milligram of betacyanin content per 100g of material weight.

$$BC\left(\frac{\text{mg}}{100\text{g}}\right) = \frac{(A_{538} \times (MW) \times V_a \times DF \times 100)}{(\epsilon \times L \times W_a)}$$

Whereas BC represented betacyanin content,  $A_{538}$  represented absorption value at 538nm, MW represented molecular weight 550g/mol,  $V_a$  represented volume of extraction solvent, DF represented diluted factor,  $\epsilon$  represented molar extinction coefficient (betanin = 60,000 L.mol<sup>-1</sup>.cm<sup>-1</sup>), L represented length of cuvette, and  $W_a$  represented weight of materials.

### 2.4 Experimentations

#### 2.4.1 Screening factors of ultrasound-assisted extraction by Plackett-Burman (P-B) design

Screening experiment was carried out with Plackett-Burman (P-B) design to determine key factors that strongly affect extraction process. Technological factors of ultrasound-assisted

extraction process including type of materials ( $Z_1$ ) (blanched and non-blanched), LS ratio ( $Z_2$ ) (mL of solvent per g of material), extraction temperature ( $Z_3$ ) ( $^{\circ}\text{C}$ ), ultrasonic time ( $Z_4$ ) (min) and ultrasound capacity ( $Z_5$ ) ( $\text{W/g}_{\text{dry weight}}$ ) were selected for this experiment. Each factor was examined at two levels (-) and (+) equivalent to low and high values. The response dependent variable of this experiment was total betacyanin content (Y).

#### *2.4.2 Optimization of the ultrasound-assisted extraction by Box-Behnken (B-B) design.*

From the previous results, key technological factors were selected for studying optimization of ultrasound-assisted extraction process.

Response surface method with Box-Behnken (B-B) design was employed instead of P-B designs because some main factors confounded with certain factors interaction and P-B designs do not have enough runs to detect an important effect. In this experimental design, B-B design envelope a factors space with three level of each factor applied (-1, 0, +1), whereas -1 and +1 are axial points and 0 is a central point (mid-range) [12].

### **2.5 Statistical analysis.**

The raw data was calculated by MS Excel 2016 and JMP version 10 was used for both P-B (Screening factors) and B-B (RSM) experimentation design with multiple regression and graphical analysis of the experimental data.

In RSM modeling, two concepts must be added to the complete mathematical model. The first is lack of fit, this term expresses the fact that the model was chosen by the experimenter before the trials is probably a little different from the true model of the studied phenomenon. There is a difference between these two models called lack of fit. The second concept is the random nature of response. In reality, measuring the same response several times at the same experimental point does not give the exactly the same result. There is a dispersion of the results called pure error [13]. The lack of fit value of predicted model is significant  $F < 0.0001^*$  mean the model is adequate to experimental.

## **3 RESULTS AND DISCUSSION**












### **3.1 Screening factors of ultrasound-assisted extraction by Plackett-Burman design**

The results of the screening experiment by P-B design are shown in Table 1. The statistical analysis results are shown in Table 2.

**Table 1:** Experimental design matrix with observed results

Run	Matri x	Type of materials (Z <sub>1</sub> )	Liquid:Solid Ratio (Z <sub>2</sub> )	Extraction temperature (°C) (Z <sub>3</sub> )	Ultrasoni c time (min) (Z <sub>4</sub> )	Power (W/g <sub>dry</sub> weight) (Z <sub>5</sub> )	Betacyanin (mg/100g)	TPC (mgGAE/g)
1	-- +-	Blanched	10 : 1	50	6	25	17.91	1.49
2	-- --	Blanched	10 : 1	70	3	25	19.84	1.03
3	-- -+	Blanched	10 : 1	70	3	75	16.10	1.31
4	+- -+	Blanched	30 : 1	50	3	75	21.01	0.58
5	+- ++	Blanched	30 : 1	50	6	75	18.61	1.01
6	+- +-	Blanched	30 : 1	70	6	25	13.02	1.84
7	+- -+	Non- blanched	10 : 1	50	3	75	17.91	0.49
8	+- +-	Non- blanched	10 : 1	50	6	25	19.81	0.960
9	+- ++	Non- blanched	10 : 1	70	6	75	16.05	0.35
10	++ --	Non- blanched	30 : 1	50	3	25	25.36	1.06
11	++ --	Non- blanched	30 : 1	70	3	25	19.46	0.75
12	++ ++	Non- blanched	30 : 1	70	6	75	16.75	0.35

**Table 2:** Statistical results of screening factors of UAE

Factors	Contrast	Plot of t-Ratio	Lenth t-Ratio	p-Value
Z <sub>3</sub>	-1.61		-42.76	< .0001*
Z <sub>4</sub>	-1.46		-38.70	< .0001*
Z <sub>5</sub>	-0.75		-19.79	< .0001*
Z <sub>1</sub>	0.74		19.54	< .0001*
Z <sub>2</sub>	0.55		14.50	< .0001*
Z <sub>3</sub> * Z <sub>4</sub>	-0.39*		-10.27	< .0001*
Z <sub>3</sub> * Z <sub>5</sub>	0.74*		19.51	< .0001*
Z <sub>4</sub> *Z <sub>5</sub>	1.12*		29.70	< .0001*
Z <sub>3</sub> * Z <sub>1</sub>	0.06*		1.60	0.1076
Z <sub>4</sub> *Z <sub>1</sub>	0.71*		18.68	< .0001*
Z <sub>5</sub> *Z <sub>1</sub>	-0.09*		-2.45	0.0222*

Statistical results show that the factors with the individual p-value smaller than 0.05 and largest plot of t-ratio were considered as significant factors [12]. All five factors showed significant effect on betacyanin content in the extract, among them Z<sub>3</sub> was the strongest factor that affect betacyanin content, followed by Z<sub>4</sub> and Z<sub>5</sub>. The Z<sub>1</sub> and Z<sub>2</sub> were the weakest factors that affected betacyanin content.

Based on screening results, three factors Z<sub>3</sub>, Z<sub>4</sub>, and Z<sub>5</sub> were chosen for further optimization of betacyanin extraction process using B-B design. The less significant factors were LS ratio (Z<sub>2</sub>) and type of material (Z<sub>1</sub>) were eliminated from B-B design, however Z<sub>2</sub> and Z<sub>1</sub> were kept constant as 30 : 1 and the blanched type, respectively for the optimization experiment using RSM. The range of Z<sub>3</sub>, Z<sub>4</sub>, and Z<sub>5</sub> values for B-B design was analyzed and chosen by JMP software.

### 3.2 Optimization of the ultrasound-assisted extraction by Box-Behnken design.

The B-B design matrix of factors and the results are shown in Table 3.

**Table 3:** Levels of factors in optimization of UAE

Factors	Levels		
	–	0	+
Ultrasonic time (min) (X1)	3.5	4	4.5
Power of ultrasound (W/g <sub>dryweight</sub> ) (X2)	50	55	60
Extraction temperature (°C) (X3)	52	53.5	55

**Table 4:** Box Behnken design matrix and observed results.

Run	Matrix	Ultrasonic time (min) (X1)	Power of ultrasound (W/g <sub>dryweight</sub> ) (X2)	Extraction temperature (°C) (X3)	Betacyanin (mg/100g) Y1	TPC (mg GAE/g) Y2
1	– – 0	3.5	50	53.5	17.99	0.387
2	– 0 –	3.5	55	52	19.12	0.390
3	– 0 +	3.5	55	55	18.17	0.403
4	– + 0	3.5	60	53.5	17.78	0.390
5	0 – –	4	50	52	18.81	0.367
6	0 – +	4	50	55	12.55	0.381
7	0 0 0	4	55	53.5	13.23	0.593
8	0 0 0	4	55	53.5	14.45	0.552
9	0 0 0	4	55	53.5	20.20	0.578
10	0 + –	4	60	52	14.36	0.460
11	0 + +	4	60	55	13.36	0.472
12	+ – 0	4.5	50	53.5	11.13	0.290
13	+ 0 –	4.5	55	52	7.47	0.214
14	+ 0 +	4.5	55	55	10.71	0.234
15	+ + 0	4.5	60	53.5	7.82	0.222

Data were analyzed through multiple regression analysis and the second order polynomial equation was obtained by JMP software. Estimates of coefficients are shown in Table 5 and the equation describing the amount of betacyanin obtained during extraction (1) (with the coefficient of determination  $R^2 = 0.95$ ) was obtained as follow:

$$Y_1 = 19.981 - 4.493X_1 - 0.894X_2 - 0.623X_3 - 0.777(X_1 \times X_2) + 1.048(X_1 \times X_3) + 1.313(X_2 \times X_3) - 3.603X_1^2 - 2.698X_2^2 - 2.511X_3^2 \quad (1)$$

**Table 5:** Statistics summary of optimization of betacyanin UAE by Box Behnken

	Estimate	Std. Error	t-Ratio	P-value
Intercept	19.981	0.35	57.07	< 0.0001*
$X_1$	-4.493	0.21	-20.96	< 0.0001*
$X_2$	-0.894	0.21	-4.17	0.0002*
$X_3$	-0.623	0.21	-2.91	0.0063*
$X_1 \times X_2$	-0.777	0.30	-2.56	0.0149*
$X_1 \times X_3$	1.048	0.30	3.45	0.0015*
$X_2 \times X_3$	1.313	0.30	4.33	0.0001*
$X_1^2$	-3.603	0.32	-11.42	< 0.0001*
$X_2^2$	-2.698	0.32	-8.55	< 0.0001*
$X_3^2$	-2.511	0.2	-7.96	< 0.0001*
N = 45	$R^2 = 0.95$	$R^2 \text{Adj} = 0.94$	$P < 0.05$	
Lack of fit: $F < 0.0001^*$		Pure Error = 2.41		

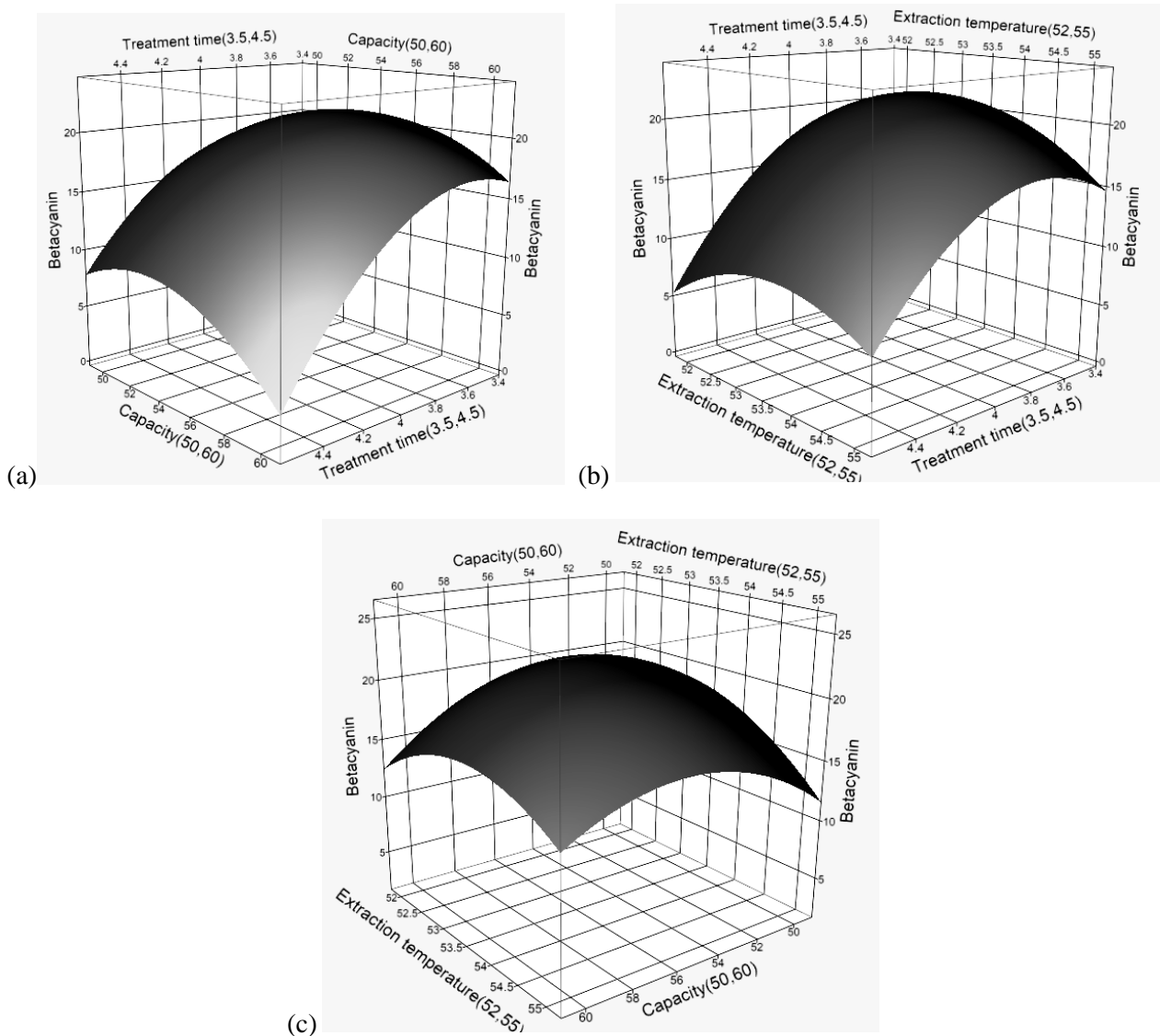
As shown in table 5, all factors showed significant effect on betacyanin content in the extract with  $p < 0.001$ . The intercept of model was positive, the first-order coefficients ( $X_1, X_2, X_3$ ) as well as their quadratic ( $X_1^2, X_2^2, X_3^2$ ) had significant negative effects on the dependent variable. That means the excessive increase of  $X_1, X_2, X_3$  led to the decrease of betacyanin concentration of the extract. Among three independent variables,  $X_1$  was the strongest factor that affected the extraction process of betacyanin. The estimates of first-order and quadratic coefficients of  $X_1$  were -4.493 and -3.603 respectively ( $p < 0.0001$ ).

The results also show that there was interaction of  $X_1 \times X_2$  (negative coefficient,  $p < 0.05$ ), and more significant interactions were found between  $X_1 \times X_3$  and  $X_2 \times X_3$  (negative coefficients,  $P < 0.01$ ). Previously, Sang et al [9] reported similar observation of the interactions of ultrasonic treatment time ( $X_1$ ) and ultrasound capacity ( $X_2$ ) on betacyanin content and extraction yield.

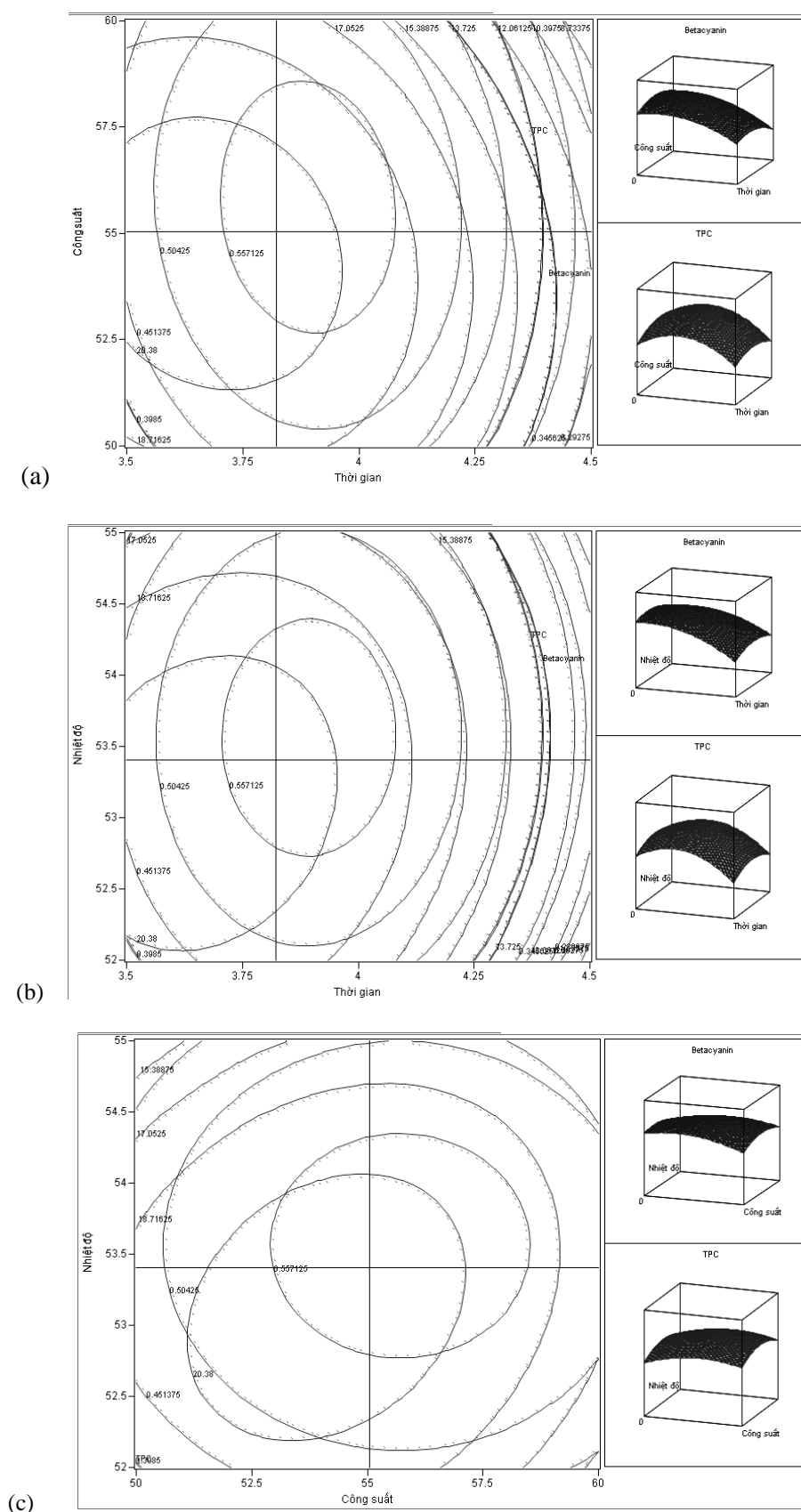
The lack of fit of the model equation was smaller than 0.0001 ( $F < 0.0001$ ), which indicated that the model was completely correlated with experimental. Using analysis of variance, the  $R^2$  value of the model was determined to be 0.94, which also showed that the regression models defined well the true behavior of the system.



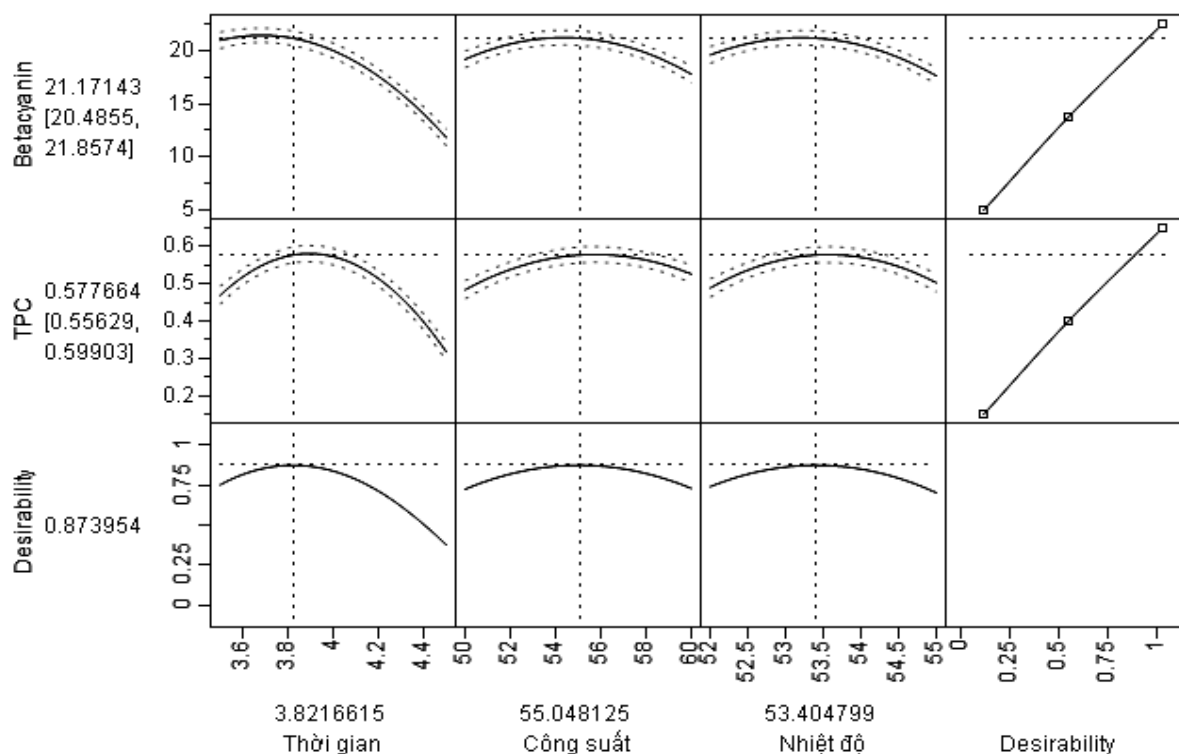
From the regression equation (1), the three dimension surface profiles and two dimension contour profiles are shown in Figure 1 and Figure 2 and the predicted values of betacyanin content in the extract were determined by JMP software (Figure 3).



**Figure 1:** Response surface profiler of the interactive effect of process variables on responses  $Y_1$  and  $Y_2$ .  
(a)  $X_1$  and  $X_2$  on  $Y_1$  and  $Y_2$ ; (b)  $X_1$  and  $X_3$  on  $Y_1$  and  $Y_2$ ; (c)  $X_2$  and  $X_3$  on  $Y_1$  and  $Y_2$



**Figure 2:** Response contour profiler of the interactive effect of process variables on responses  $Y_1$  and  $Y_2$ . (a)  $X_1$  and  $X_2$  on  $Y_1$  and  $Y_2$ ; (b)  $X_1$  and  $X_3$  on  $Y_1$  and  $Y_2$ ; (c)  $X_2$  and  $X_3$  on  $Y_1$  and  $Y_2$ .



**Figure 3:** Prediction profiler of two responses  $Y_1$  and  $Y_2$

Figure 3 show that, the increase of ultrasonic treatment time and ultrasound capacity led to the increase of betacyanin content in a short period. The cavity formation and the collapse of cavitation bubbles on a material's surface occur when ultrasound pass through medium results in micro-jetting which generates several effects such as surface peeling, erosion and particle breakdown, leading to higher mass transfer and increase of extraction rate and yield [7]. When ultrasonic treatment time was over 3 minutes 45 seconds and the ultrasound capacity was over 55W/g<sub>dry weight</sub>, the betacyanin content began to decrease. This can be explained that betacyanin in the extract was probably oxidized by the action of free radicals produced by ultrasonic wave at high power and longtime treatment.

The extraction temperature also had a great influence on betacyanin content of the extract. When temperature was 53°C, the betacyanin content reached highest value. However when temperature was above 53°C, the betacyanin content decreased sharply. This can be explained by the temperature sensitivity of the pigment. Temperature increases the diffusion of betacyanin into the extract but high temperature causes the degradation of betacyanin.

Based on the predicted model, the optimum conditions of extraction process were found as follows: ultrasonic time 3.82 minutes (3 minutes 49 seconds), ultrasound capacity 55.05 W/g dry weight, extraction temperature 53.4°C. Predicted maximum betacyanin content was 21.171 mg/100g<sub>dry weight</sub>.

The optimal parameters were verified. The results of verification experiment are shown in Table 6. The experimental values were therefore similar to the predicted value from equation (1). The deviation of model was only 1.99%.

**Table 6:** Verified the model of ultrasound-assisted extraction

	Betacyanin (mg/100g)	TPC (mg GAE/g)
Predicted responses of model	21.17	0.577
Verified responses of model	$21.17 \pm 0.500$	$0.580 \pm 0.01$
Deviation of model	1.99%	0.51%

## 4 CONCLUSIONS

Plackett-Burman experimental design and response surface method (RSM) were successfully applied to determine optimum technological parameters for betacyanin extraction from dragon fruit peel. The optimal UAE conditions were obtained as follows:

- Ultrasonic time: 3.82 minutes (3 minutes 49 seconds)
- Ultrasound capacity: 55.05 W/gdry weight
- Extraction temperature: 53.4°C

Maximum betacyanin content in the extract was 21.171 mg/100g dry weight, similar to the predicted value from regression equation.

Further research should pay attention on the combination of ultrasound with other techniques such as enzymatic treatment to increase the extraction yield. In addition, pigments stability and betacyanin color preservation studies will also be carried out.

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## **EFFECT OF PHOSPHATES AND TREATMENT CONDITIONS ON QUALITY AND SAFETY OF FROZEN FILLET TRA FISH (*PANGASIANODON HYPOPHthalmus*)**

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### **ABSTRACT**

Polyphosphates are legally permitted additives that are widely used to aid in processing for improving eating quality of many foods, particularly meat and fish products. Usage of polyphosphate to fish before freezing often helps to reduce the amount of thaw drip that is the liquid releasing when frozen fish is thawed. Applying a large amount of polyphosphate in fish processing is occurring in a lot of frozen fish companies which can become a negative factor to the development of exported products. The objective of this study was to determine the effects of various phosphate blends and treatment conditions (method, temperature, and time of tumbling) on the quality and safety of frozen Tra fillets in Mekong Delta, Vietnam. Tra fillets were dipped in brine solutions consisting of 0.5% NaCl and 1.1% phosphate for 15 minutes. Three kinds of phosphate solutions were applied for this experiment: (i) sodium tripolyphosphate (STPP), (ii) mixture of STPP + sodium polyphosphate, and (iii) STPP + sodium polyphosphate + sodium diphosphate. The ratio of Tra fillets and solution was 2:1. In addition, the optimal temperature for phosphate treatment was observed. Fillets were evaluated for drip loss, tumbling yield, color, pH, tenderness, water holding capacity, and sensory properties. It was found that STPP gave a significant lower drip loss ( $P < 0.05$ ) and higher pH value than other treatments. All phosphate treatments significantly decreased ( $P < 0.05$ ) the tenderness, especially STPP blends significantly ( $P < 0.05$ ) improved the lightness of the product. When temperature conditions in a range of 6÷12°C were applied for phosphate treatment, there was not significant ( $P < 0.05$ ) change in the yield and the quality of Tra fillets. Accordingly, the treatment of Tra fillet with solution of STPP at 9÷12°C was chosen.

**Keywords:** drip loss, hardness, phosphate, Tra fillets, water uptake.

## 1 INTRODUCTION

Tra or Pangasius (*Pangasianodon hypophthalmus*) has been raised mainly in Lao, Vietnam, Cambodia and Thailand, however, the aquaculture industry has been only developed rapidly in Vietnam, especially in the Mekong delta. Vietnamese Pangasius is consumed mainly in the form of frozen fillets for export. Up to now, frozen Tra fillets have been introduced to 131 countries and territories globally. According to the Ministry of Agriculture and Rural Development, in 2017, export of Tra fish reached 1.78\$ billion, up 4.3% compared to 2016, contributing nearly 22% to the total export value of fisheries. Frozen Tra fillets of Vietnam have also become a brand name product on the market of catfish world and the third most widely sold aquaculture fish product on world markets after salmon and tilapia.

To improve sensory properties as well as reducing drip loss when thawing frozen fish, phosphate food additives are applied with different levels in many Tra fish processing establishments. Despite bearing many commercial names, the widely used phosphate additives were divided into 3 categories: (1) additives containing only sodium tripolyphosphate (E 451); (2) additives containing sodium tripolyphosphate (E 451) and sodium polyphosphate (E 452); (3) additives containing sodium diphosphate (E 450), sodium tripolyphosphate (E 451), and sodium polyphosphate (E 452). There is considerable controversy about mechanisms of phosphate functionality, particularly as it relates to enhance water holding capacity in meats, and fish [1, 2]. Some hypothetical factors discussed among several researchers have shown that the actions of the polyphosphates in the muscular tissue can happen due to (a) the increase of the pH of the meat, (b) the increase of the ionic force, (c) the chelating of metallic ions, and (d) the dissociation of the actomyosin complex [3]. The application of phosphates in frozen Tra fillet processing is shown promisingly; however, abusing phosphates can make the impact on the quality of treated fillets. Phosphates increase pH of fish products, however, if the final pH of the product is high, the shelf life decreases and failures due to sliminess, translucency and fat decomposition [1, 3]. Moreover, when high levels of polyphosphates are used, the processing and the flavor of products can be affected. Polyphosphate can be hydrolyzed to the orthophosphate form in the presence of the phosphatase enzyme. If that happens, the orthophosphates can react with the fatty acid to form soap presenting a specific flavor [3]. According to Aitken (2001) [4], the excessive treatment of small products such as thin fillets could result in undesirable flavor changes and sloppy texture. Phosphates can also be misused to retain excessive moisture in frozen fish fillets. Such an excessive water adds to total weight of the final product, resulting in misrepresentation when frozen fish prices are charged for water. So, the incorrect or abusive use of phosphates can characterize economic frauds. Therefore, the objective of the study is to investigate the impact of phosphate additives, solution concentration, and treatment conditions on the quality, and food safety of frozen Tra fillets.



## 2 MATERIALS AND METHODS

### 2.1 Materials

708 alive Tra fish (*Pangasianodon hypophthalmus*) with the harvest size of 900 to 1,100 g were selected from random farms in the Mekong delta.

Experiments were carried out at a fish processing factory in accordance with the following process: Alive fish receiving → Slaughtering → 1<sup>st</sup> washing → Filleting → Skinning → 2<sup>nd</sup> washing → Trimming → 3<sup>rd</sup> washing → Coding, weighing, whiteness measuring → 4<sup>th</sup> washing, temperature adjusting → Additive treating (fish fillets, and solution were poured into PE bags, the ratio of fillets and solution was 2:1. Then the bags were sealed, and put into a stainless steel tumbling drums, and tumbled together with other fillets of the factory at 7 rounds per minute) → 5<sup>th</sup> washing, draining, weighing (for determination of water uptake, or weight gain) → Freezing → Glazing → Packaging → Storing (at  $-20 \pm 2^{\circ}\text{C}$  for 3 weeks) → Sampling and analyzing.

### 2.2 Experimental setup

#### 2.2.1 Exp. 1: The effect of types of phosphate additive on quality of frozen Tra fillets

The experiment was conducted as a randomized complete block design with one factor. Treatments were replicated 3 times. Three types of commercial phosphate additives (Add.1 contains E 451; Add.2 contains E 451, and E 452; Add.3 contains E 450, E 451 and E 452) at the concentration of 1.1% (based on  $\text{P}_2\text{O}_5$ ) combined with 0.5% NaCl were investigated. Each treatment consisting of 8 fillets and was done at  $9 \pm 0.5^{\circ}\text{C}$  for 15 minutes. The final products were thawed, and tested for drip loss, texture (hardness and cutting test), pH, color (whiteness change), and sensory properties (appearance and flavor).

#### 2.2.2 Exp. 2: The effect of pre-treatment temperature on water uptake, water holding capacity and texture of frozen Tra fillets

The experiment was conducted as a randomized complete block design with one factor. Treatments were replicated 3 times. Eight fillets of each treatment were treated by solution consisting of 2% of the additive chosen from experiment 1 and 0.5% NaCl at 4 levels of initial temperature of fillets, ranged from 6 to  $15^{\circ}\text{C}$ , for 15 minutes. The final products were thawed and tested for drip loss, water holding capacity (WHC), and texture properties (hardness and cutting test).

#### 2.2.3 Exp. 3: The effect of phosphate additive concentration and tumbling time on quality of frozen Tra fillets

The experiment was conducted as a randomized complete block design with two factors. Treatments were replicated 3 times. Twenty-four fillets of each treatment were treated with four concentration levels of the additive chosen from experiment 1, ranged from 1 to 4%, for 4 levels



of tumbling time, ranged from 10 to 25 minutes, at the temperature based on the result of experiment 2. The final products were thawed and tested for drip loss, pH, phosphate content, and hardness.

### 2.3 Testing method

*Thawing and draining method:* Frozen fillets were packaged in watertight sealed PE bags, and put in a water bath, water volume of the fish was about 8:1, at  $25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ . The fillets were immersed in the water bath until reaching to the unfrozen state. Thawed fillets then placed on the wall of plastic sieves for 5 minutes.

Weight gain (%) =  $(\text{Weight after treatment} / \text{Weight before treatment} - 1) \times 100$

Net weight gain (%) =  $(\text{Weight after thawing} / \text{Weight before treatment} - 1) \times 100$

Drip loss (%) =  $(1 - \text{Weight after thawing} / \text{Net weight before thawing}) \times 100$

*Whiteness change measurement:* Each fillet was measured for whiteness (expressed as W index) on 6 different positions by using Color Reader Whiteness Index (Model CR-14, Minolta, Japan).

**Whiteness change (%) =  $(\text{W after thawing} / \text{W before treatment} - 1) \times 100$**

*Texture evaluation:* The texture of samples was objectively measured by using a texture analyzer (TA-XT2i, Stable Micro System, UK) with following compression test parameters: load cell = 25kg, probe = P75 cylinder for hardness measurement, and probe = HDP/BSK Blade set with knife for cutting test, test speed = 2mm/s and distance = 60%. The texture property – hardness is defined as the peak force of the first compression of the sample. For cutting test, the maximum force denotes the point at which the sample completely fills with the blade, and the sample surface was cut through. For each result, 6 samples were measured, and an average value was calculated.

*WHC evaluation:* The WHC of fish samples was determined by using the filter paper press method (FPPM) [5].

*pH measurement:* pH of fish was measured by ISO 2917:1999 method [6]

*Phosphate content determination:* Phosphate content of fish was determined by NMKL (No. 57-1994) method [7].

*Sensory evaluation:* Thawed fish fillets were evaluated by a trained sensory panellist for appearance (wholeness, defects such as sliminess, and translucency) and flavor. To determine flavor, fillets were cut in 5x5cm, and poured into PE bags with NaCl 0.8%, then boiled for 12 minutes. The results were expressed in 6 levels of point and decreased from 5 to 0 corresponding with the quality decline.

## 2.5 Data analysis

All data were statistically analyzed, the analysis of variance and Duncan's Multiple Range Test was applied to assess the difference between means, processed by Statgraphics Centurion 16.2 ( $p \leq 0.05$ ) and Excel 2016 programs.

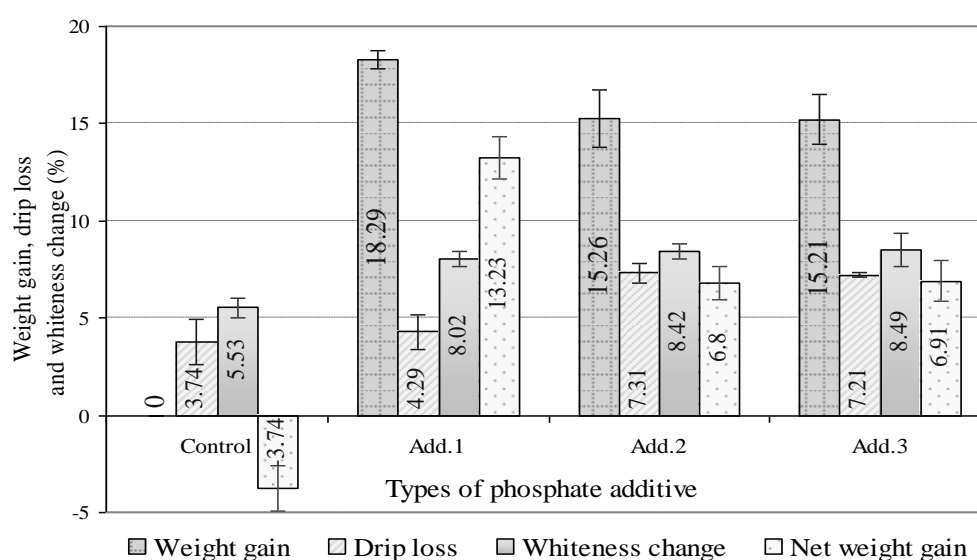
## 3 RESULTS AND DISCUSSION

### 3.1 The influence of types of phosphate additive on quality changes of frozen Tra fillet

**Table 1:** Effect of types of phosphate additive on quality of frozen Tra fillets; different letters in the same columns indicate significant differences in the test treatments at 95% confidence intervals; values are given as means  $\pm$  stdev

Additive	pH	Texture		Sensory properties	
		Hardness (g)	Cutting test (g)	Appearance (point)	Flavor (point)
Control	6.45 <sup>a</sup> $\pm$ 0.04	7188 <sup>a</sup> $\pm$ 522	1301 <sup>a</sup> $\pm$ 149	4.73 <sup>a</sup> $\pm$ 0.12	4.53 <sup>a</sup> $\pm$ 0.31
Add.1	6.81 <sup>c</sup> $\pm$ 0.06	5190 <sup>b</sup> $\pm$ 270	962 <sup>b</sup> $\pm$ 92	4.27 <sup>b</sup> $\pm$ 0.12	4.53 <sup>a</sup> $\pm$ 0.12
Add.2	6.61 <sup>b</sup> $\pm$ 0.04	5399 <sup>b</sup> $\pm$ 532	1080 <sup>b</sup> $\pm$ 38	4.33 <sup>b</sup> $\pm$ 0.12	4.40 <sup>a</sup> $\pm$ 0.15
Add.3	6.74 <sup>bc</sup> $\pm$ 0.11	5032 <sup>b</sup> $\pm$ 265	1007 <sup>b</sup> $\pm$ 96	4.13 <sup>b</sup> $\pm$ 0.31	4.27 <sup>a</sup> $\pm$ 0.31

Add.1 contains E 451; Add.2 contains E 451 and E 452; Add.3 contains E 450, E 451 and E 452)



**Figure 1:** The effect of phosphate types to the changes of weight gain, drip loss and whiteness of Tra fillet

(Add.1 contains E 451; Add.2 contains E 451 and E 452; Add.3 contains E 450, E 451 and E 452)

The influences of types of phosphate additive on the quality value of thawed Tra fillets were determined. The water uptake, drip loss, and whiteness change of fillet sample is shown in Figure 1, while texture, pH and sensory properties of frozen Tra fillet are presented in Table 1. Based on the result in Figure 1 and Table 1, usage of different types of phosphate gave a significant ( $P < 0.05$ ) effect on quality of frozen Tra fillet sample. The first and universal effect of all polyphosphate treatment observed was the increase in the weight of the fish by retaining water from 15.21 to 18.29% comparing to the control.

In Figure 1, although drip loss of sample treated phosphate was higher than the control, many samples increased net weight gain comparing to the initial sample. This demonstrated that the main value of polyphosphates gains in improving the retention of water by protein in fish [4].

In addition, the whiteness of treated fillet samples was also improved in both three types of phosphate additives, and the control. This could be attributed to drip loss associated with frozen storage [8]. Myoglobin and pigment compounds in muscle can be released with thaw drip. In case of phosphate treatment, a large of water in muscle protein may be the main cause for whiteness improvement of thawed fish fillet. In addition, polyphosphate treatment can improve the appearance of prepacked chilled fillets by preventing the accumulation of unsightly drip within the pack [4], this can help to explain the increase of whiteness of Tra fillet. However, polyphosphate treatment before freezing could only reduce the loss, and this does nothing to prevent the corresponding deterioration in flavor and texture [1, 4]. The decrease of hardness, and cutting force also found in phosphate treated sample (Table 1). Types of polyphosphate gave different changes in quality of Tra fillet after freezing and thawing. Because the content of  $Mg^{2+}$ , and  $Ca^{2+}$  in Tra fish were low (about 12 mg Mg/100 g and 10 mg Ca/100 g Tra fillet [9, 10], the water remaining in the fish fillet surface seemed to depend on the high pH value of phosphate solution. When Tra fish was treated by STPP (additive 1 – E 451), a higher value of pH was obtained ( $6.81 \pm 0.06$ ) comparing to the others (6.61 and 6.74). Accordingly, a decrease in drip loss and an increase in of weight gain were observed.

It can be said that sodium tripolyphosphate (E 451) is an optimal type of phosphate additive for pretreatment Tra fillet before freezing. However, other factors of pre-treatment process, such as the treated temperature, treatment time and the concentration of E 451 also give strong influence on quality changes of frozen Tra fillets.

### **3.2 The influence of pre-treatment temperature on physical chemical properties of frozen Tra fillet**

The effect of temperature of phosphate treatment process on the physical properties of Tra fillet was determined and the result was summarized in Table 2.

**Table 2:** Influence of pre-treatment temperature on quality changes of Tra fillet; different letters in the same columns indicate significant differences in the test treatments at 95% confidence intervals; values are given as means  $\pm$  stdev

Pre-treatment temperature	WHC (%)	Weight gain (%)	Drip loss (%)	Texture	
				Hardness (g)	Cutting test (g)
6 $\pm$ 0.5°C	51.47 <sup>a</sup> $\pm$ 0.62	17.90 <sup>a</sup> $\pm$ 0.72	4.50 <sup>a</sup> $\pm$ 0.72	4664 $\pm$ 222 <sup>a</sup>	1,101 $\pm$ 19 <sup>a</sup>
9 $\pm$ 0.5°C	51.50 <sup>a</sup> $\pm$ 0.72	17.82 <sup>a</sup> $\pm$ 1.15	4.32 <sup>a</sup> $\pm$ 0.27	4450 $\pm$ 217 <sup>a</sup>	1,098 $\pm$ 35 <sup>a</sup>
12 $\pm$ 0.5°C	51.26 <sup>a</sup> $\pm$ 0.54	17.61 <sup>a</sup> $\pm$ 0.74	4.31 <sup>a</sup> $\pm$ 1.12	5066 $\pm$ 635 <sup>a</sup>	1,139 $\pm$ 43 <sup>a</sup>
15 $\pm$ 0.5°C	48.93 <sup>b</sup> $\pm$ 0.76	17.56 <sup>a</sup> $\pm$ 1.66	4.41 <sup>a</sup> $\pm$ 1.11	4221 $\pm$ 566 <sup>a</sup>	1,069 $\pm$ 81 <sup>a</sup>

When applying different pre-treatment temperature for tumbling Tra fillets in STPP solution, there was non-significant ( $P > 0.05$ ) change in physical properties of all treated samples. However, for the pre-treatment Tra fillet at the temperature of 15°C in STPP solution, the WHC of fish protein had a lower value than other treatment temperature (in a range of 6 to 12°C). This result demonstrated that the high temperature (15°C in this case) of pre-treatment process may cause the increase in the protein denaturation leading to the decrease in WHC [10]. Thus, the optimal temperature for phosphate treatment process can be in a range of 6 to 12°C. However, the range of 9 to 12°C was chosen for treatment process of Tra fillet in solution of STPP to reduce energy consumption.

### 3.3 The influence of phosphate solution concentration and time of tumbling

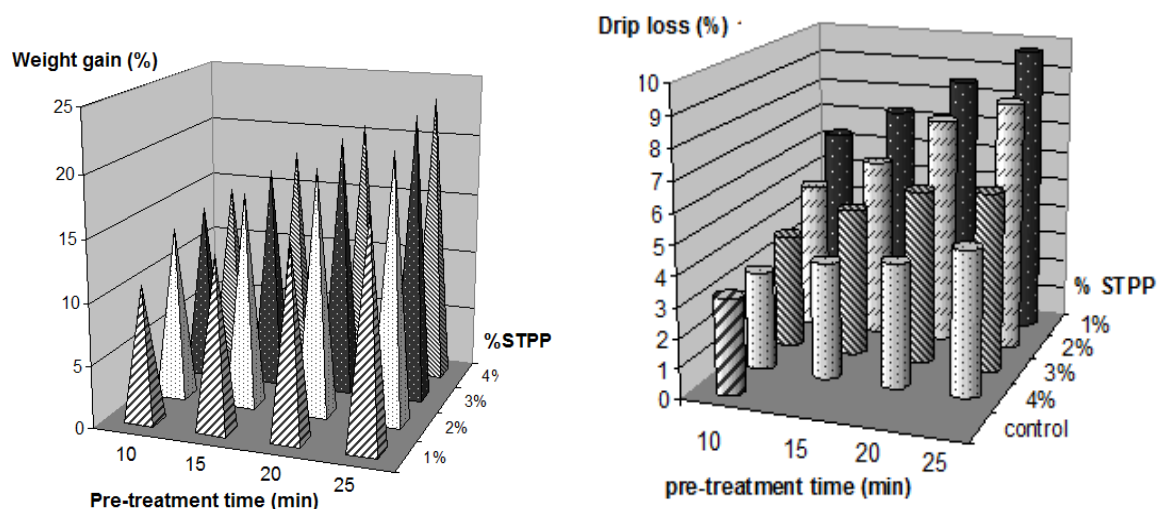
The pH value, hardness, and content of  $P_2O_5$  in the Tra fillet sample at different time of tumbling in 4 levels of phosphate concentration were showed in the Table 3.

**Table 3:** Influence of phosphate solution and time of tumbling on the changes of pH, hardness and  $P_2O_5$  content in Tra fillet; different letters in the same columns indicate significant differences in the test treatments at 95% confidence intervals; values are given as means  $\pm$  stdev

STPP concentration (%)	Time of tumbling (min)	pH	$P_2O_5$ (%)	Hardness (g)
Control	0	6.60 <sup>a</sup> $\pm$ 0.05	0.31 <sup>a</sup> $\pm$ 0.02	6219 <sup>a</sup> $\pm$ 674
1%	10	6.63 <sup>ab</sup> $\pm$ 0.06	0.34 <sup>b</sup> $\pm$ 0.01	6044 <sup>ab</sup> $\pm$ 597
	15	6.66 <sup>abc</sup> $\pm$ 0.04	0.34 <sup>b</sup> $\pm$ 0.01	5928 <sup>abc</sup> $\pm$ 319
	20	6.73 <sup>cde</sup> $\pm$ 0.03	0.35 <sup>b</sup> $\pm$ 0.01	5783 <sup>abcd</sup> $\pm$ 187
	25	6.78 <sup>e</sup> $\pm$ 0.01	0.37 <sup>b</sup> $\pm$ 0.01	5396 <sup>de</sup> $\pm$ 140
2%	10	6.70 <sup>bcd</sup> $\pm$ 0.10	0.41 <sup>c</sup> $\pm$ 0.01	5569 <sup>bcd</sup> $\pm$ 354

STPP concentration (%)	Time of tumbling (min)	pH	P <sub>2</sub> O <sub>5</sub> (%)	Hardness (g)
3%	15	6.75 <sup>de</sup> ± 0.06	0.44 <sup>cd</sup> ± 0.02	5516 <sup>cde</sup> ± 261
	20	6.88 <sup>f</sup> ± 0.05	0.45 <sup>d</sup> ± 0.01	5397 <sup>de</sup> ± 206
	25	6.92 <sup>fg</sup> ± 0.02	0.47 <sup>de</sup> ± 0.02	4850 <sup>fgh</sup> ± 219
	10	6.89 <sup>f</sup> ± 0.01	0.49 <sup>e</sup> ± 0.01	5325 <sup>def</sup> ± 235
	15	6.93 <sup>fg</sup> ± 0.03	0.53 <sup>f</sup> ± 0.03	5036 <sup>efg</sup> ± 144
	20	6.94 <sup>fgh</sup> ± 0.02	0.55 <sup>fg</sup> ± 0.02	4869 <sup>fgh</sup> ± 139
4%	25	6.98 <sup>ghi</sup> ± 0.04	0.58 <sup>g</sup> ± 0.02	4370 <sup>hi</sup> ± 279
	10	6.93 <sup>fg</sup> ± 0.02	0.49 <sup>e</sup> ± 0.02	4790 <sup>gh</sup> ± 223
	15	6.97 <sup>ghi</sup> ± 0.05	0.53 <sup>f</sup> ± 0.04	4752 <sup>ghi</sup> ± 295
	20	7.01 <sup>hi</sup> ± 0.06	0.56 <sup>fg</sup> ± 0.03	4375 <sup>hi</sup> ± 223
	25	7.03 <sup>i</sup> ± 0.06	0.63 <sup>h</sup> ± 0.02	4242 <sup>i</sup> ± 149

Based on the data in Table 3, it can be seen that when tumbling Tra fillets was in STPP solution at higher concentration (> 2%), the pH value of samples increased. In case of 1% STPP, there was a significant ( $P < 0.05$ ) increase of pH observed at the longer treatment time (20 and 25 mins). In Figure 2, it is obvious that the decrease in drip loss and increase in weight gain occurred. This demonstrated that there was a relationship between concentration and time of additive treatment process of Tra fillet. However, the value of P<sub>2</sub>O<sub>5</sub> exceeded the limited level of 0.5% [11] when the treatment time was so long (more than 15 min), and the used concentration of STPP solution was over 3%. Besides, hardness of frozen Tra fillets decreased significantly ( $P < 0.05$ ) when high solution concentration and long treatment time were applied.



**Figure 2:** The weight gain and drip loss (%) of thawed Tra fillet in pre-treatment condition

## 4 CONCLUSIONS

The application of polyphosphate treatment is needed for sensory especially color improvement, and nutrition loss prevention in frozen Tra fillet processing. Types of polyphosphate, and pre-treatment conditions (temperature, time, and concentration of additive) gave a significant effect on quality changes of frozen Tra fillets. At the same concentration, using STPP alone helped the fillets remained the best water binding comparing to other mixtures. At the moderate level, addition of STPP in frozen Tra fillets could help to reduce the drip loss by improving the water retention of the protein in fish without absorbing too much water into the final products and kept  $P_2O_5$  content lower than the maximum level.

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## **USE OF SOY PROTEIN ISOLATE AND CARBOHYDRATE FOR MICROENCAPSULATION OF RAMBUTAN SEED OIL BY SPRAY- DRYING**

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### **ABSTRACT**

In this study, rambutan seed oil was microencapsulated by spray-drying for stabilization of oil quality during the storage. Different carbohydrates including gum arabic, maltodextrin, sucrose, and glucose were alternatively combined with soy protein isolate for microencapsulation of rambutan seed oil. Mixture of soy protein and maltodextrin generated the spray-dried powder with high microencapsulation efficiency as well as high microencapsulation yield. This wall system also produced the powder with the lowest peroxide value at the end of the acceleration storage. The appropriate soy protein/maltodextrin ratio was 1/3 under which the microencapsulation efficiency and yield for rambutan seed oil were 94.7% and 71.9%, respectively. Microencapsulation was a potential method for protection of rambutan seed oil against deterioration.

**Keywords:** Maltodextrin, Microencapsulation, Rambutan seed oil, Spray-drying, Soy protein.

### **1 INTRODUCTION**

Rambutan is a tropical fruit widely cultivated in South-East Asia. Rambutan seed is a by-product in canned fruit industry. The percentage of seed in rambutan fruit varied from 7 to 10%. The seed is rich in oil content which is approximately 33.4 – 37.2% dry weight [1]. Previous studies revealed that physicochemical and thermal characteristics of rambutan seed oil may become interesting for specific applications in several segments of the food industry [2]. However, rambutan seed oil contains high level of unsaturated fatty acids including oleic acid (36.8%), linolenic acid (6.5%), linoleic acid (1.4%), erucic acid (0.7%) and palmitoleic acid (0.5%), which are highly susceptible to microbiological, chemical and biochemical deteriorations. Therefore, prevention of rambutan seed oil during the preservation is essential [1, 2]. Lipid microencapsulation by spray-drying is a well-known method in food industry. In this method, lipid (core material) is packaged within a wall material and solid powder of micro particles is obtained. This method involves three basic steps: mixing lipid and wall solution for preparation



of an oil-in-water emulsion, homogenization and spray-drying of the emulsion with hot air for formation of lipid powder [3].

Criteria for wall materials used in oil microencapsulation consisted of high emulsifying properties, high water solubility, low viscosity and high film-forming properties [4]. Proteins including sodium caseinate, whey protein, soy protein isolate and gelatin exhibited desirable characteristics of wall materials [3, 5]. Carbohydrates are generally added as a secondary wall material (a filler) to improve drying properties of sprayed droplets by enhancing the formation of dry crust around drying droplets and increase the oxidative stability of microencapsulated oils by reducing oxygen permeability of wall matrix [6, 7]. Carbohydrates used in lipid microencapsulation can be divided into two major groups: low molecular weight carbohydrates (glucose, maltose, lactose, maltodextrin) and high molecular weight carbohydrates (modified starch, gum arabic) [4, 7]. Nevertheless, comparison of carbohydrates used in combination of soy protein isolate for lipid microencapsulation has not been reported.

In this work, rambutan seed oil was microencapsulated by spray-drying. Soy protein isolate was combined with different carbohydrates as wall systems. The objective of this study was to select the appropriate carbohydrate in combination with soy protein isolate in the microencapsulation of rambutan seed oil.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Raw rambutan seeds were collected from a canned fruit processing plant in Dong Nai province. The seeds were washed with water and manually separated the kernels. The kernels were dried at  $40^{\circ}\text{C} \pm 2^{\circ}\text{C}$  to a moisture content of approximate 10% and ground down. Oil extraction from the kernels was performed with hexan under the following conditions: material and solvent ratio of 1:10 (w/w), temperature of  $40^{\circ}\text{C}$  and time of 36h. After extraction, the liquid phase was separated by filtration. Hexan was removed from the rambutan seed oil by distillation at  $60^{\circ}\text{C}$ . The physico-chemical characteristics of rambutan seed oil were as follows: moisture content:  $0.2 \pm 0.01\%$ ; acidic value:  $0.35 \pm 0.02$  mg KOH/kg oil; peroxide value:  $4.25 \pm 0.02$  meq/kg oil; iodine value:  $41.6 \pm 0.3$  g/100g oil.

Soy protein isolate (Protein content: 90% on dry basis) was supplied by Foodchem International. Gum Arabic (Purity degree: 99.5%, heavy metals: less than 20ppm) was originated from Jumbo Acacia Co., Ltd (ThaiLand). Maltodextrin (Dextrose equivalent: 12, heavy metals: less than 5ppm) was supplied by Roquette Frères (France). Sucrose (Purity degree: 99.9%, ash: 0.05%) was purchased from Bien Hoa Sugar Jointstock Company (Vietnam). Glucose (Purity degree: 99.5%, heavy metals: less than 5ppm) was originated from P.S.C. Starch Product PLC (Thailand).

All solvents and chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich (The United States).

## **2.2 Methods**

### *2.2.1 Procedure of rambutan seed oil microencapsulation*

Microencapsulation of rambutan seed oil was performed as follows: 10% (w/w) protein solution (De Barros Fernandes et al., 2014) was prepared by dissolving protein preparation in distilled water, stirring at 50°C and 750 rpm for 6 h on C-MAG HS4 heating magnetic stirrer (IKA, Malaysia). Carbohydrate was then dissolved in the protein solution; the weight ratios of protein/carbohydrate in the wall solution was 1/1. Rambutan oil was then added to protein solutions and the total solid of the mixtures obtained was 20% (w/w). The weight ratio of lipid to protein was therefore 1:1 [8]. All samples were treated with Heidolph Diax 900 mechanical homogenizer (Labexchange, Germany) at 8000 rpm for 5 min and with high-pressure homogenizer Model 1000 (APV, Denmark) at 200 bar for 3 recirculation [9]. Finally, emulsion samples were spray – dried by a Mobile Minor-Model E spray-drier (Niro A/S, Denmark). The emulsions were fed into the chamber at the rate of 1.6 L/h by a 505S peristaltic pump (Matson-Marlow, England). The atomization was performed by TS Minor MO2/B rotary atomizer. The drying took place with an air inlet temperature of 180°C, air outlet temperature of 60°C [10] and air pressure of 3 bar at the atomizer. The powder of each run was collected for further analysis.

### *2.2.2 Selection of carbohydrate for combination with soy protein isolate in microencapsulation of rambutan seed oil*

In this section, different carbohydrates including gum arabic, maltodextrin, glucose, sucrose were alternatively combined with soy protein isolate for microencapsulation of rambutan seed oil. At the end of the spray-drying, powder samples were analyzed to measure total and surface oil level, acidic and peroxide value. In addition, the powder samples in high density polyethylene package with vapor-permeability of 15 g/m<sup>2</sup>.day were then stored at 60°C under vapor-saturated conditions for 30 days in order to accelerate oil oxidation. During the accelerated storage, samples were taken every five days to determine the peroxide value.

### *2.2.3 Effects of soy protein isolate/carbohydrate ratio on microencapsulation of rambutan seed oil*

Based on the results of the previous section, one carbohydrate was selected for combination with soy protein isolate for rambutan seed oil microencapsulation in this experiment. Four emulsion samples were prepared. The total solid of all emulsion samples was fixed at 15% (w/w). The weight ratio of lipid to wall material in the emulsion samples was fixed at 1/2. The weight ratio of protein to carbohydrate was 1/1, 1/2, 1/3 and 1/4. Other operating conditions were similar to those of the previous section. Powder samples were subjected to similar analyses as mentioned above.

### **2.3 Analytical methods**

Total solid content of oil-in-water emulsion (g/L) was determined by drying at  $110\pm 3^{\circ}\text{C}$  until constant weight [11]. Total oil content of oil-in-water emulsion (g/L) was determined by using a method proposed by Lakshanasomya et al. (2011) with slight modification. Ten milliliters of emulsion was taken into the oil extraction flask for analysis.

Firstly, 1.5 mL of ammonium hydroxide was added and mixed followed by 10 mL of alcohol (9%) and the contents were again well mixed. Secondly, 25 mL diethyl ether was added to the flask; it was then shook vigorously for 1 min. Finally, 25 mL of light petroleum ether (b.p.  $40\text{--}60^{\circ}\text{C}$ ) was added and the flask was shook vigorously for 1 min. After separation was complete, the oil solution was transferred into a Petri dish and the Petri dish was dried at  $102\pm 2^{\circ}\text{C}$  for 1 h and weighed. The total oil content was calculated as the difference between weight of Petri dish with oil and weight of initial Petri dish.

Viscosity of oil-in-water emulsion (cP) was measured at  $30\pm 2^{\circ}\text{C}$  using Brookfield viscometer DV1 with spindle no. 1 and rotation rate of 100 rpm. Moisture content of the oil powder (%) was evaluated by drying at  $110\pm 2^{\circ}\text{C}$  until constant weight [11].

The oil on the surface of the powder particles (g/g) was determined by a method suggested by Young, et al. [12]. One gram of the powder was accurately weighed into the extraction flask. Subsequently, 25 mL of petroleum ether (b.p.  $40\text{--}60^{\circ}\text{C}$ ) was added and the mixture was shook vigorously for 10 min. The mixture was then filtered through a cloth. The filtrate was transferred into the Petri dish, dried at  $102\pm 2^{\circ}\text{C}$  for 1 h and weighed. The surface oil content was calculated as the difference between weight of Petri dish with oil and weight of initial Petri dish.

The total oil content of the spray-dried powder (g/g) was determined by using a method described by Young et al. (1993). One gram of the powder was accurately weighed into the oil extraction flask. Water was added to complete the volume to 10 mL and mixed. The total oil content in the emulsion was determined by using a method proposed by Lakshanasomya et al. (2011).

The encapsulated oil content (g/g) was calculated as a difference of the total oil content and the surface oil content of the powder obtained. Particle size distributions of the spray-dried powder samples were analyzed by using a Model LA 920 laser diffraction particle analyzer (Horiba, Japan). A small sample was suspended in 99.5% ethanol using magnetic agitation and the distribution of particle size was monitored during three successive measurements. The volume mean diameter of spray-dried powder particles ( $\mu\text{m}$ ) was expressed as  $D_{4,3}$  (De Brouckere mean diameter).

Microencapsulation efficiency and yield were calculated by formulas reported by Shu et al. (2006). Microencapsulation efficiency was defined as a ratio between the mass of the encapsulated oil and the mass of the total oil in the spray-dried powder. Microencapsulation yield

was defined as a ratio between the mass of the total oil of the spray-dried powder and the mass of the total oil of the emulsion before spray drying.

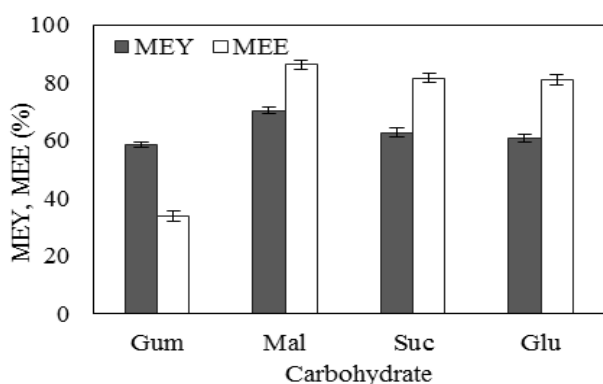
## 2.4 Statistical analysis

All experiments were performed in triplicate. Mean values were considered significantly different when  $P < 0.05$ . One-way analysis of variance was performed using the software Statgraphics Centurion X V.

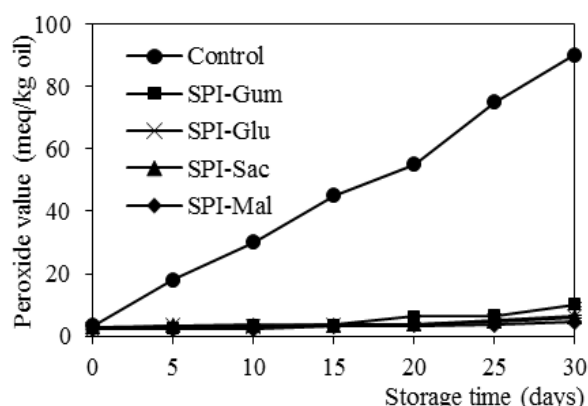
## 3 RESULTS AND DISCUSSION

### 3.1 Selection of carbohydrate for combination with soy protein isolate in microencapsulation of rambutan seed oil

Figure 1 shows that the lowest microencapsulation yield (MEY) of 58.4% was observed for the combined soy protein isolate and gum arabic. It can be explained by high viscosity of the emulsion with this mixture (Table 1). Gharsallaoui *et al.* (2007) explained that high viscosity emulsion interfered with the atomization process and led to formation of elongated droplets which adversely affected the drying rate [13]. The combination of SPI and sucrose or glucose also generated low microencapsulation yield 62.6% and 62.7% (Figure 1) although the viscosity of their original emulsions was very low (Table 1). These sugars have low glass transition temperature and that led to an enhanced stickiness of the powder particles and a high loss of the product [14]. Mixture of SPI and maltodextrin resulted in highest microencapsulation yield (70.3%). The higher the lipid microencapsulation yield, the lower the oil loss during the spray-drying and the better the recovery yield of the process.



**Figure 1:** Effects of the combination of different carbohydrates and soy protein isolate on the microencapsulation yield and efficiency of rambutan seed oil (Weight ratio of SPI/ carbohydrate in wall solution was 1/1; Gum: gum arabic, Mal: maltodextrin, Suc: sucrose, Glu: glucose)



**Figure 2:** Change in peroxide value of rambutan seed oil powder during the accelerated storage

Figure 1 also shows that the combination of SPI and low molecular carbohydrates including glucose, sucrose and maltodextrin generated very high microencapsulation efficiency (MEE) which was varied from 80.7% to 86.1%. In contrast, the combination of SPI and high molecular weight carbohydrates such as gum Arabic resulted in lower microencapsulation efficiency (33.8%). Similar results were reported in microencapsulation of pumpkin seed oil when sodium caseinate and low molecular carbohydrates or sodium caseinate and high molecular carbohydrates were used [15]. Klein et al. (2010) noted that the interaction of protein and certain carbohydrates such as gum arabic was rather weak [16] and this mixture generated non-resistant multilayer around oil droplets.

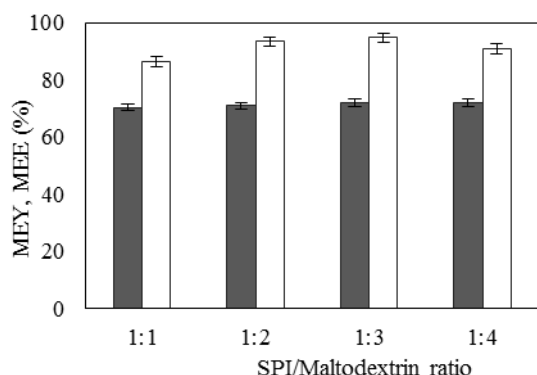
**Table 1:** Viscosity of oil – in – water emulsion before the spray – drying (The weight ratio of protein/carbohydrate in the wall solution was 1/1)

Wall material	SPI – gum arabic	SPI – maltodextrin	SPI – sucrose	SPI – glucose
Viscosity (cP)	1792.2± 79.0 <sup>a</sup>	792 ± 59 <sup>c</sup>	35.7 ± 0.8 <sup>b</sup>	31.1 ± 1.6 <sup>b</sup>

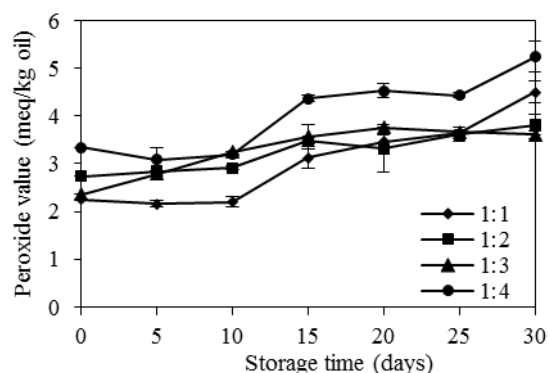
*Values with different small letters in the same row are significantly different ( $P < 0.05$ )*

Figure 2 presents the change in peroxide value of the rambutan seed oil powder during the accelerated storage. For all samples, the peroxide value gradually increased during the time due to oil oxidation. At the end of the accelerated storage, the control demonstrated the highest peroxide value of 92 meq/kg oil due to intensive oxidation of non-microencapsulated rambutan seed oil sample. On the contrary, the peroxide value of all microencapsulated powder samples slightly increased. Among the microencapsulated rambutan seed oil samples, the peroxide value of sample with soy protein isolate and gum arabic wall was the highest ( $10.1 \pm 0.9$  meq/kg oil) and that of the sample with soy protein isolate and maltodextrin wall was the lowest ( $4.3 \pm 0.7$  meq/kg oil). According to Gharsallaoui (2007), maltodextrin provide good oxidative stability to encapsulated oil but exhibit poor emulsifying capacity, emulsion stability and low oil retention. In contrast, the protein such as soy protein isolate, sodium caseinate, and whey protein concentrates have an amphiphilic character that offer physicochemical and functional properties required to encapsulate hydrophobic core material [13]. Therefore, the combination of SPI and maltodextrin was the best protective ability against oil oxidation, highest microencapsulation yield and efficiency, so that it was used as wall material in the next experiment.

### 3.2 Effects of soy protein isolate/ maltodextrin ratio on microencapsulation of rambutan seed oil



**Figure 3:** Effects of the weight ratio of SPI/maltodextrin in wall system on the microencapsulation yield (MEY - black bars) and microencapsulation efficiency (MEE - white bars) of rambutan seed oil



**Figure 4:** Effects of the weight ratio of SPI/maltodextrin in wall solution on the change in peroxide value of rambutan seed oil powder during the accelerated storage

Figure 3 shows that the weight ratio of soy protein isolate/ maltodextrin (DE 12) changed from 1/1 to 1/4 did not affect the microencapsulation yield. Similar observation was also noted when the ratio of whey protein/ maltodextrin (DE 12) was varied from 1/1 to 1/4 in rambutan seed oil microencapsulation [17].

Figure 3 also reveals that reduction in soy protein isolate/maltodextrin ratio from 1/1 to 1/3 increased the microencapsulation efficiency from  $86.1\% \pm 1.2\%$  to  $94.7\% \pm 0.9\%$ . Nevertheless, further reduction in soy protein isolate/maltodextrin ratio from 1/3 to 1/4 slightly decreased the microencapsulation efficiency.

Effects of the weight ratio of soy protein isolate/maltodextrin in wall system on the change in peroxide value of rambutan seed oil powder during the accelerated storage are presented in Figure 4. After 30 days of the accelerated storage, the peroxide value of all samples slightly increased. Among them, the peroxide value of the weight ratio 1/4 sample was the highest ( $5.2 \pm 0.7$  meq/kg oil). Both samples with the soy protein isolate/maltodextrin ratio of 1/2 and 1/3 resulted in statistically similar peroxide value ( $3.6 \pm 0.2$  meq/kg oil). The cost of maltodextrin was much lower than that of SPI. The appropriate soy protein isolate/ maltodextrin ratio was therefore 1/3.

## 5 CONCLUSIONS

When the soy protein isolate has been combined with carbohydrate, the microencapsulation efficiency for rambutan seed oil was effected by carbohydrate molecular weight. Higher microencapsulation efficiency achieved when soy protein isolate was combined with lower molecular weight carbohydrate. Soy protein isolate and maltodextrin resulted in high

microencapsulation efficiency (94,7%) and yield (71,9%) and the appropriate soy protein isolate/maltodextrin ratio was 1/3. The combination of protein and low molecular weight carbohydrate for lipid microencapsulation was a potential method for protection of rambutan seed oil against deterioration.

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## ULTRASOUND-ASSISTED EXTRACTION OF PROTEIN FROM BRACKISH-WATER ALGAE *CHAETOMORPHA* SP.

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### ABSTRACT

The protein of biomass *Chaetomorpha* sp. was extracted with sodium hydroxide solvent using ultrasound-assisted method. The effects of ultrasound-assisted extraction conditions on the protein yield were alternatively investigated. At the solvent:material ratio of 4:1 (v:w), NaOH concentration of 0.75% (w/v); ultrasonic power of 30 W per gram of material; ultrasonication temperature of 50°C and treatment time of 2 min, the protein yield archived 72% and this value was 50% higher than that in the conventional extraction with ultrasonic treatment. The extraction rate constant of the ultrasound-assisted extraction was 20% higher than that of the conventional extraction.

**Keywords:** *Chaetomorpha*, kinetic parameters, protein extraction, ultrasound-assisted.

### 1. INTRODUCTION

*Chaetomorpha* sp. (*Chlorophyta*) is one of sea algae genus which has been distributed along the coast of Vietnam. Due to climate change, sea level has risen and saltwater has moved into the Mekong River Delta. These phenomena generated the brackish ecosystem. *Chaetomorpha* sp. has been found to be widely distributed in shrimp (crab) pond in Mekong delta. *Chaetomorpha* biomass is a potential feed for aquatic animals. However, in the rainy season, intensive growth of the algae is observed and that decreased the oxygen content for aquatic animal growth. In Mekong delta, *Chaetomorpha* biomass has been as waste for environmental pollution. It was reported that *Chaetomorpha* biomass contained high protein level (12-17% dry base) and the content of essential amino acids achieved 42,1% of total protein [1]. It would be a potential source for animal nutrition.

Protein concentrates have been used as nutritional supplement and functional ingredient in food and feed processing. Extraction is a key operation in the production of protein concentrates. Since

the last decade, ultrasound-assisted extraction has attracted great attention. Use of ultrasound significantly enhanced the protein extraction yield from soy meal [2], rice brane [3], pumpkin seed meal [4]... Nevertheless, ultrasound-assisted extraction of protein from *Chaetomorpha* biomass has never been reported.

In this study, ultrasound was applied to protein extraction from *Chaetomorpha* sp. biomass. The effects of conditions of the ultrasound-assisted extraction on the protein yield were investigated. In addition, kinetic parameters of the ultrasound-assisted extraction and the conventional extraction were also evaluated and compared.

## **2. MATERIALS AND METHODS**

### **2.1 Raw material**

*Chaetomorpha* sp biomass was obtained from the brackish-water aquaculture ponds in Bac Lieu province (Vietnam). Fresh algae biomass was crushed in a miller to 2 mm size particles and preserved at -60°C in the freezer for experimentation. Chemical composition (% dry weight) of *Chaetomorpha* biomass was as follows: 12-14% protein, 38-45% cellulose, 1.9-2.1% lipid and 27-32% ash.

### **2.2 Conventional extraction of protein**

The conventional extraction was carried according to method of Barbarino & Lourenco [5] with slight modifications. Five gram fresh algae biomass was mixed with 20 mL 0.5% (w/v) NaOH solution in 100 mL erlenmeyer flask. Protein extraction was conducted in an thermostat shaker at 50°C, 150 rpm for 1 hour and then centrifuged at 21°C, 15,000 g for 20 min. The pellet was discarded and the supernatant was used for protein concentration determination.

### **2.3 Ultrasound-assisted extraction of protein**

Five gram fresh *Chaetomorpha* biomass was mixed with 20mL 0.5% (w/v) NaOH solution. The ultrasound-assisted extraction consisted of two steps: ultrasonic treatment and additional extraction. For the first step, the sample in 100 mL erlenmeyer flask was treated with an ultrasonic probe. During the ultrasonic treatment, the erlenmeyer flask was immersed in a cooling bath for control of extraction temperature. For the second step, the erlenmeyer flask was incubated in a thermostat shaker at 50°C and 150 rpm as mentioned in the conventional extraction. Various technological factors of the ultrasound-assisted extraction were investigated.

*First series:* The ratio of solvent:material was changed from 2:1 to 6:1. The concentration of NaOH solution, ultrasonic power, temperature and time were 1.0% (w/v), 25 W/g material, 30°C and 1 min, respectively.

*Second series:* The sodium hydroxide concentration was changed: 0.5, 0.75, 1.0, 1.25, 1.5 and 2.0% (w/v). The ratio of solvent:material was selected from the results of first series. The ultrasonic power, temperature and time were 25 W/g material, 30°C and 1 min, respectively.

*Third series:* The ultrasonic power was changed: 20, 25, 30, 35 and 40 W/g material. The ratio of solvent:material and sodium hydroxide concentration were selected from the result of first and second series, respectively. The ultrasonic temperature and time were 30°C, 1 min, respectively.

*Fourth series:* The ultrasonic temperature was varied: 30, 40, 50, 60 and 70°C. The ratio of solvent:material, sodium hydroxide concentration and ultrasonic power were selected from the result of first, second and third series, respectively. Ultrasonic treatment time was 1 min.

*Fifth series:* The ultrasound-assisted extraction of protein was carried in 1 to 6 min with results were selected from all above series.

## 2.4 Analytical methods

Protein content of the algae biomass and the extract was determined by Kjeldahl method.

Particles size distribution of the algae biomass at the end of the conventional and ultrasound-assisted extraction was determined using laser diffraction particle size distribution analyzer HORIBA LA-920 (Japan). The samples were mixed with water at concentration of 0.05% (w/v).

Morphology of the material particle was observed by scanning electron microscopy (Jeol JSM 7401F, Germany)

## 2.5 Kinetic parameters of the conventional and ultrasound-assisted extraction of protein

The first-order kinetic model of conventional and ultrasound-assisted extraction was used for determination of the extraction rate constant of protein [6]. The general first-order model was as follow:

$$(C_{\infty} - C_t)/(C_{\infty} - C_w) = e^{-kt} \quad (1)$$

where,  $C_{\infty}$  is maximal protein concentration in the extract (g/L),  $C_t$  is protein concentration in the extract at a given extraction time  $t$  (g/L),  $C_w$  was initial protein concentration in the extract (g/L),  $k$  was extraction rate constant (g/L.min).

Due to  $C_w = 0$  when  $t = 0$ , the first-order model can be written as equation (2):

$$(C_{\infty} - C_t)/C_{\infty} = e^{-kt} \quad (2)$$

The integrated rate law for a first-order extraction under the boundary conditions  $t = 0$  to  $t$  and  $C_t = 0$  to  $C_t$  can be written as equation (3):

$$d(C_t)/dt = d(C_{\infty} \times (1 - e^{-kt}))/dt$$

$$d(C_t)/dt = k \times C_{\infty} \times e^{-kt} \quad (3)$$

When  $t = 0$ , initial extraction rate  $h$  (g/L.min) can be defined as:

$$h = k \times C_{\infty}$$

The maximal protein concentration in the extract  $C_{\infty}$  (g/L), initial extraction rate  $h$  (g/L.min) and extraction rate constant  $k$  (g/L.min) were determined by using R software (version x64 3.4.1).

The protein yield of the extraction was calculated by the following formula:

$$Y = P_a/P_t$$

Where:  $Y$  (%) was the protein yield,  $P_a$  (g) was the total protein content in the extract,  $P_t$  (g) was the total protein content in algae biomass used in the protein extraction.

## 2.6 Statistical analysis

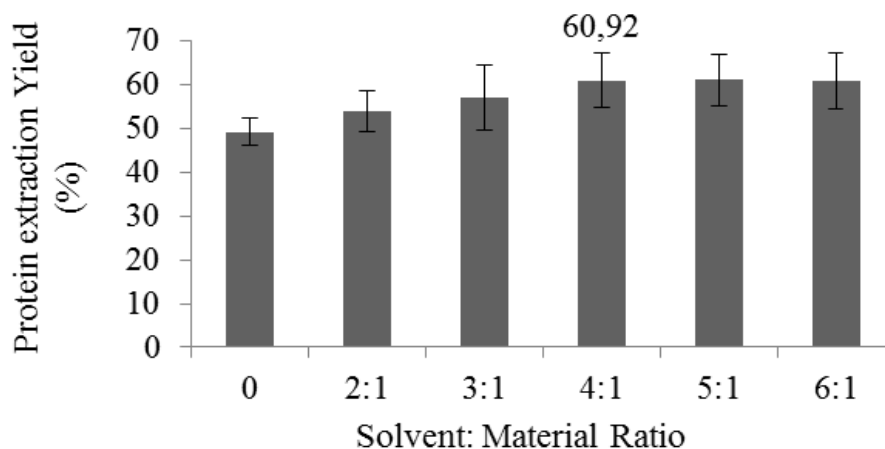
All experiments were performed in triplicate. The experimental results were expressed as means  $\pm$  standards deviation. Mean values were considered significantly different when  $p < 0.05$ . One-way analysis of variance was performed using the software Statgraphics Centurion XV.

## 3 RESULTS & DISCUSSION

Glutelin is the main protein fraction in most green algae [7]. Sodium hydroxide solution was therefore selected as solvent for protein extraction from *Chaetomorpha* sp. biomass.

### 3.1 The effects of the solvent:material ratio

Figure 1 presents the effects of solvent:material ratio on the protein extraction yield. When the solvent/material ratio increased from 2:1 to 4:1, the protein yield increased from 54.0 to 60.9%. Higher solvent:material ratio did not change protein yield. It can be explained that high solvent ratio increased the mass transfer in liquid-solid extraction [8] and that enhanced the protein extraction yield. Nevertheless, increase in solvent:material from 4:1 to 6:1 did not improve the protein yield. The solvent:material ratio of 4:1 was therefore selected for protein extraction from *Chaetomorpha* sp. biomass.

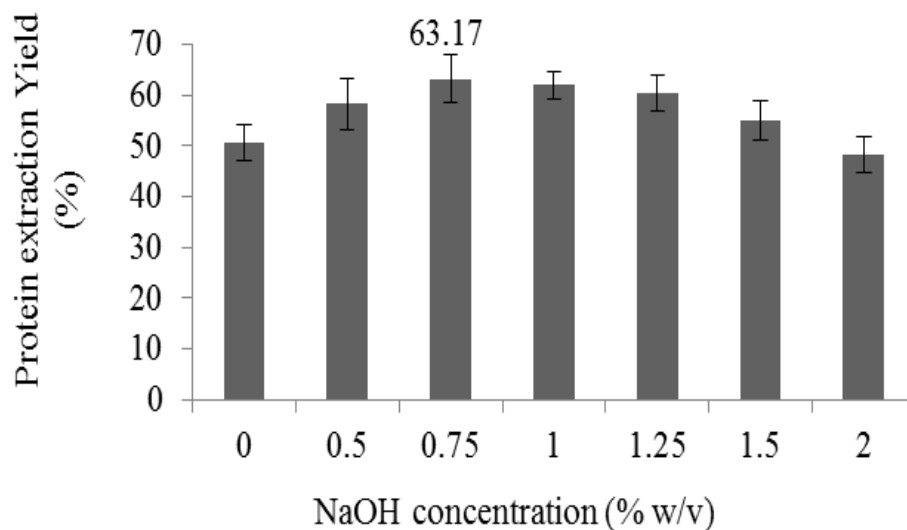


**Figure 1:** The effect of the solvent:material ratio on the protein yield

### 3.2 The effect of sodium hydroxide concentration

Figure 2 shows the protein extraction yield at different sodium hydroxide concentrations. Increase in sodium hydroxide concentration from 0.5 to 0.75% increased the protein yield from 58.22 to 63.17 %. It can be explained that cellulose is the main component of cell wall while protein is located inside algae cells. Degradation of cell wall results in better protein extraction. According to Zhang *et al.* [9], crystalline structure of cellulose was partially damaged under alkaline condition.

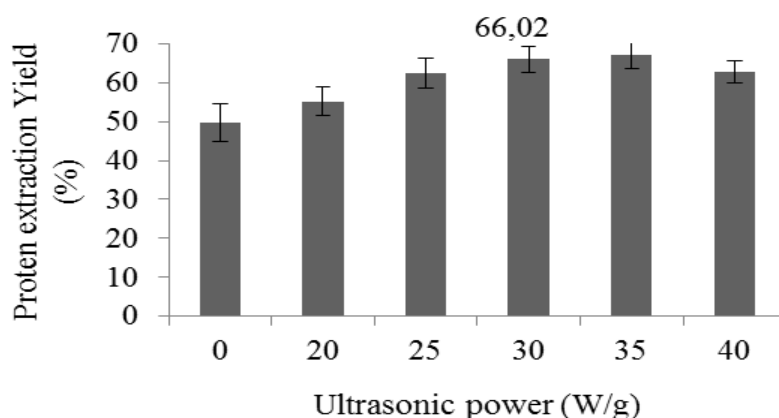
The higher sodium hydroxide concentration, the higher the number of damaged cells and the protein yield was improved. Nevertheless, at the higher sodium hydroxide concentration, the protein yield was gradually decreased due to denaturation of protein molecules. Similar observation was reported by Florence [10]. The sodium hydroxide concentration of 0.75 % was therefore selected for algae protein extraction.



**Figure 2:** Effect of sodium hydroxide concentration concentration on the protein yield

### 3.3 Effect of ultrasonic power

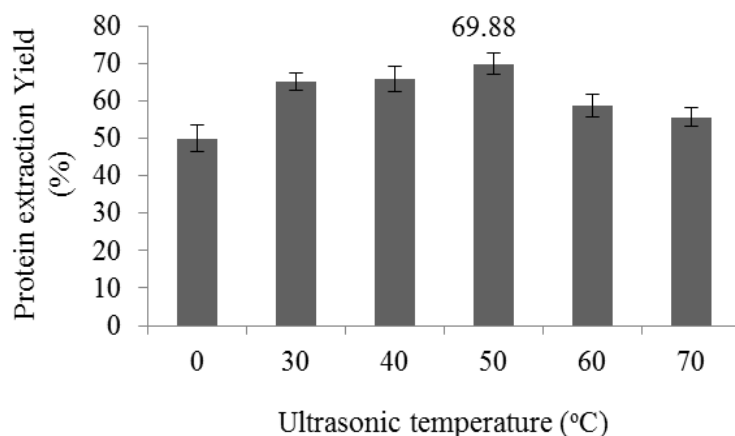
Figure 3 presents the effects of sonication power on the protein yield. All samples treated with ultrasound had significantly higher protein yield than the control sample. It can be concluded that use of ultrasound clearly enhanced the protein extraction from *Chaetomorpha* sp.. When the ultrasonic power increased from 20 W/g to 30 W/g material, the protein yield increased by 35%. Increase in ultrasonic power from 30 to 35 W/g did not change the protein yield. Nevertheless, higher ultrasonic power slightly reduced the protein yield. Ultrasound generated acoustic cavitation which enhanced mass transfer in solid-liquid extraction [11]. This phenomenon could improve the protein extraction yield. The higher the ultrasonic power, the more intensive acoustic cavitation and the better the protein extraction. However, so high ultrasonic power generated free radicals with high level. These free radicals might react with sulfhydryl groups of protein molecules resulting in protein denaturation [12]. That was the main reason for decreased protein yield at high ultrasonic power. Tu *et al.* [4] previously reported that increase in ultrasonic power from 20 to 25 W/g reduced the protein extraction yield by 7 % from pumpkin seed.



**Figure 3:** Effect of ultrasonic power on the protein yield

### 3.4 Effect of ultrasonic treatment temperature

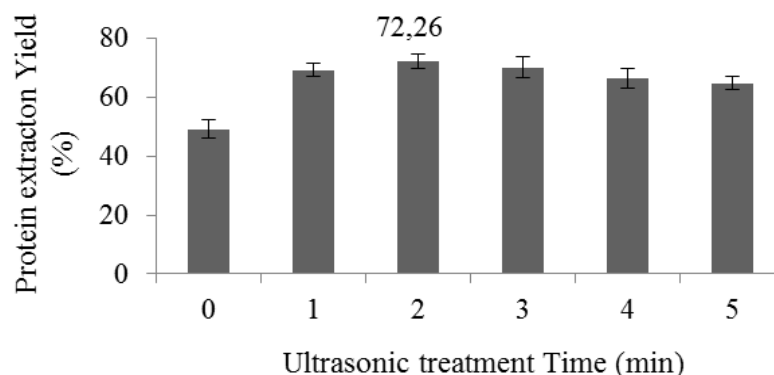
The effects of ultrasonic temperature on the protein yield is shown in figure 4. As the temperature rise from 30°C to 50°C, the protein yield increased from 65.11 to 69.88%. Increased temperature reduced viscosity in solid-liquid extraction which facilitated the formation and explosion of gas bubbles [11]; acoustic cavitation was therefore intensive and that improved the protein extraction yield. At higher temperatures (60 and 70°C), the protein yield significantly decreased probably due to protein denaturation. However, too high temperature will denature protein and reduce extraction efficiency. Similar effects of ultrasonication temperature on the protein extraction from almond dregs were reported by Zhang *et al.* [9].



**Figure 4:** Effect of ultrasonic temperature on the protein yield

### 3.5 Effect of ultrasonication time

Figure 5 shows that increase in ultrasonication time from 0 to 2 min augmented the protein extraction yield by 53%. Longer treatment time decreased the protein yield. Prolong ultrasonication led to an intensive accumulation of free radicals [13] and that may resulted in denaturation of soluble protein in the extract. This result was consistent with the findings of Wang [14] on ultrasound-assisted extraction of protein from soybean flakes.

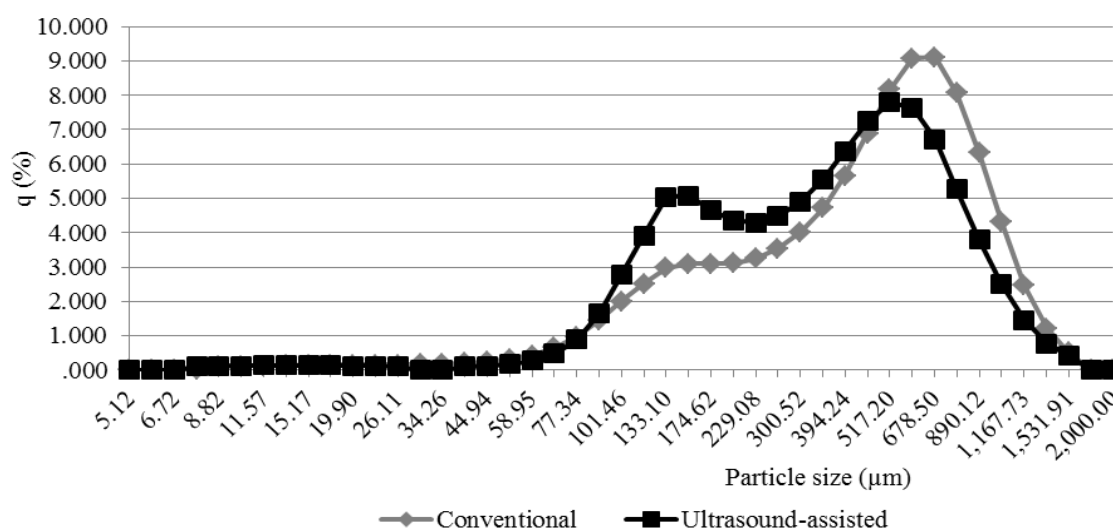


**Figure 5:** Effect of ultrasound treatment time on the protein yield

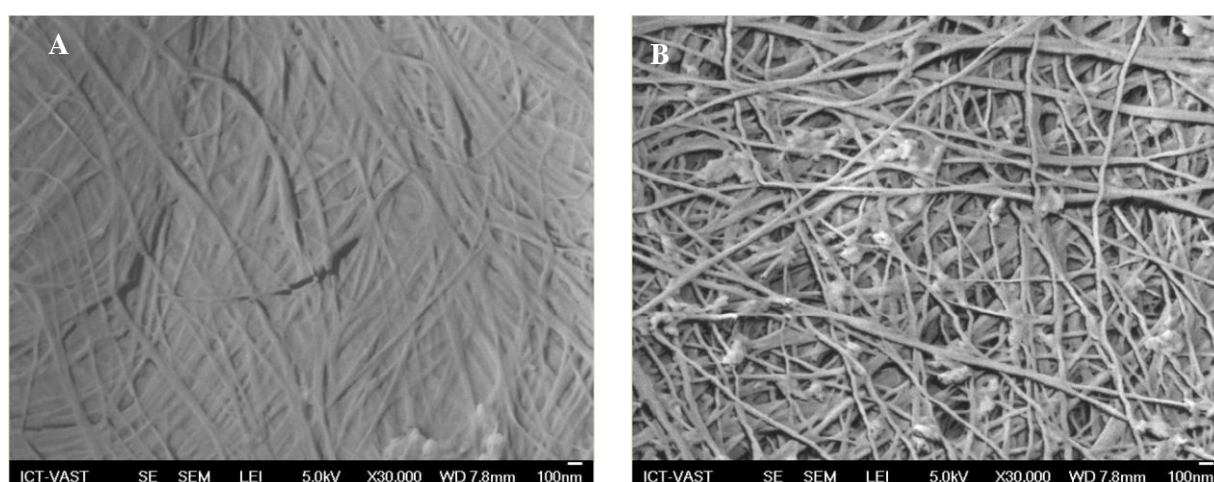
### 3.6 Structure analysis of the material

Figure 6 presents particle size distribution of the solid material at the end of the conventional and ultrasound-assisted extraction. One main peak at 678.5  $\mu\text{m}$  was observed for the conventional method while two peaks at 517.2 and 152.45  $\mu\text{m}$  were detected for the ultrasonic method. In addition, the average size of solid material at the end of the extraction was 467.78  $\mu\text{m}$  and 392.59  $\mu\text{m}$  for the conventional and ultrasound-assisted extraction, respectively. As a consequence, ultrasonic treatment significantly decreased the material particle size and that highly enhanced the protein extraction due to high contact surface between solid material and liquid solvent.





**Figure 6:** PSD of the material at the end of the conventional and ultrasound-assisted extraction. The ultrasonic power, temperature and time were 30 W/g, 50 °C and 2 min, respectively

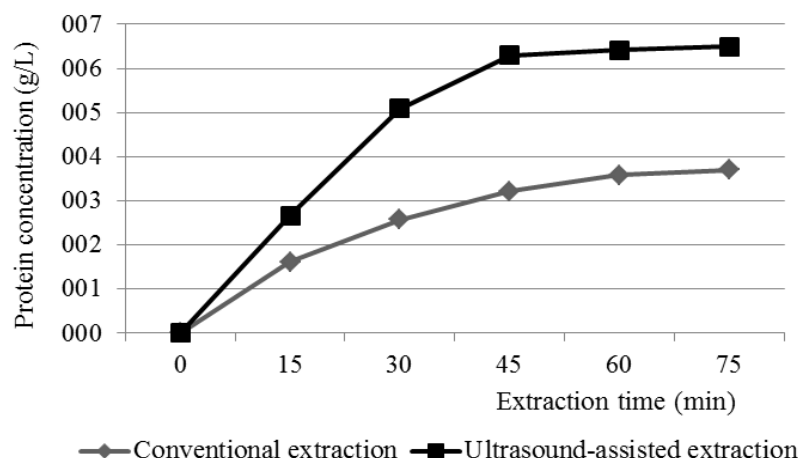


**Figure 7:** SEM images of the material particle at the end of the conventional (A) and ultrasound-assisted extraction (B). The ultrasonic power, temperature and time were 30 W/g, 50 °C and 2 min, respectively

Figure 7 presents surface of the material particle at the end of the protein extraction. For the conventional extraction, cellulose fibrils were orderly arranged (Fig 7A) and that resulting in high resistance of cellulose crystal structure. Nevertheless, the particle surface in the ultrasound-assisted extraction was highly porous. This observation reveals that the surface of solid material was highly damaged during the ultrasound-assisted extraction. Consequently, solvent diffusion into the material particle was improved and protein extraction was enhanced.



### 3.7 Kinetic parameters of classical and ultrasound-assisted protein extraction



**Figure 8:** Change in protein concentration in the extract during the ultrasound-assisted and conventional extraction

Figure 8 shows the change in protein concentration of the extract during the conventional and ultrasound-assisted extraction. Based on the obtained results, the maximal protein concentration in the extract  $C_{\infty}$  (g/L), initial extraction rate  $h$  (g/L.min), extraction rate constant  $k$  (g/L.min) and coefficient of determination  $R^2$  were determined and shown in Table 1. The coefficient of determination  $R^2$  for both conventional and ultrasound-assisted extraction was very high. It can be concluded that the first order kinetic model describes well the experimental results in our study. Previously, first order model was also used to calculate kinetic parameters of protein extraction [6, 3].

According to the model, the maximum protein concentration in the extract in the ultrasound-assisted method was 1.73 times higher than that in the conventional method. In addition, the initial extraction rate ( $h$ ) and extraction rate constant ( $k$ ) of the ultrasound-assisted extraction were 2.07 times and 1.20 times, respectively higher than those of the conventional extraction. That was due to an improved mass transfer in ultrasonic extraction in comparison with that in conventional extraction [11]. Higher extraction rate led to a shorter extraction time.

**Table 1:** Comparison of the first-order kinetic parameters of the conventional and ultrasound-assisted extraction of protein from *Chaetomorpha* sp. biomass

Extraction method	Extraction capacity ( $C_{\infty}$ )	Initial extraction rate ( $h$ )	Extraction rate constant ( $k$ )	$R^2$
Conventional	3.800	0.152	0.040	99.36
Ultrasound-assisted	6.570	0,315	0.048	97.97

## 4 CONCLUSIONS

Ultrasound-assisted extraction has been shown to be an efficient method for the extraction of protein from *Chaetomorpha* sp. biomass. Use of ultrasound resulted in higher concentration of protein in the extract as well as shorter extraction time in comparison with the conventional extraction. A considerable reduction in the extraction time seems to be suitable for the extraction of thermally labile proteins from plant material.

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## OPTIMIZATION OF SALT - WATER SUPPLEMENTATION COMBINED WITH OTHER ADDITIVES TO IMPROVE THE QUALITY OF SNAKEHEAD FISH CAKE

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### ABSTRACT

The research was conducted to determine the role of some food additives in the quality of fried fish cake processed from cultured snakehead fish (*Channa striata*) surimi. The study investigated the effect of modified starch supplementation on the quality of fried fish cake made from snakehead fish surimi and also evaluated the interaction between salt and water addition to product quality. At the same time, the study examined the effect of supplemental chitosan on the gel and microbiological properties of this product. Results showed that the addition of 3% modified starch in the cutting process helped improve the quality of fried fish cake from snakehead fish surimi. The study confirmed that there was a correlation between the water and salt addition to fried fish cake quality, the optimum percentages of water and salt was 6.17% and 1.32%, respectively. According to the study, the addition of chitosan at a ratio of 75 mg/100 g of surimi in the cutting process resulted in improved gel properties and limited microbial growth.

**Keywords:** Surimi, modified starch, NaCl, chitosan, snakehead fish cake.

### 1 INTRODUCTION

Snakehead fish have long been known to be a good source of protein, especially for people with poor health. The consumption of snakehead fish can help support wound healing, analgesic and additional energy during the recovery process [1]. These values are due to the adequate supply of fish protein, the balance of essential and non-essential amino acids [2]. As a result of the washing process, surimi has the advantages that other foods do not have, such as high fiber protein content, good structural properties, and low lipid, so it is suitable for the processing of gel product [3]. Surimi is only a semi-finished fish product that is stored frozen and used as a raw material for the processing of fish cakes, fish balls and other simulated products before consumption.

Fish cake, a protein-based fish product with a long history of development, is very popular, utility and highly nutritious. Fish cake made from snakehead fish surimi promises to be a good source of

protein for people with diabetes, obesity and those susceptible to fishy smell. However, for good quality fish cakes, especially gel structure and prolonged shelf life, the use of suitable additives is usually very important. In particular, the use of salt not only has a positive effect but also increase protein's water holding capacity, thereby improving the structure of emulsified products [4]. Borderias et al. [5] proposed the use of modified starches in combination with plant proteins to improve the structural properties of protein-based products from fish, shrimp. Recently, Prabpree and Pongsawatmanit [6] and Cierach et al. [7] also confirmed the role of modified starches in improving water holding capacity and structural stability of fish cakes. In addition, chitosan is thought to help improve the gel system while preventing the growth of microorganisms in the product, thus extending the shelf life of surimi. Kungsuwan et al. [8] showed that chitosan solution played a very important role in improving the gel properties of Thai catfish (*Pangasius suichi*) surimi.

From the above studies, the establishment a process of processing fish cakes from snakehead fish surimi by supplementary additives that improve structural properties and prolong storage capacity was deployed.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

The research was conducted at the Laboratory of Food Technology, Faculty of Agriculture and Applied Biology, Can Tho University. Major equipment and chemicals used in the study include Planimeter (HAFF No. 317 E, Germany), Colorimeter (NH300 D65, China), RheoTex (SD305, Japan).

- Modified starch (Acetylated di-starchadipate, France), Sorbitol (Neo sorbitol, France), Sodium Chloride (China), Sodium tripolyphosphate (France).
- Chitosan oligomer, supplied by Chitosan Vietnam Company (manufactured in Go Dac village, Binh An commune, Chau Thanh district, Kien Giang province).
- Pepper, garlic, sugar and MSG are supplied from supermarkets in Can Tho City, according to Vietnamese standards for food processing.

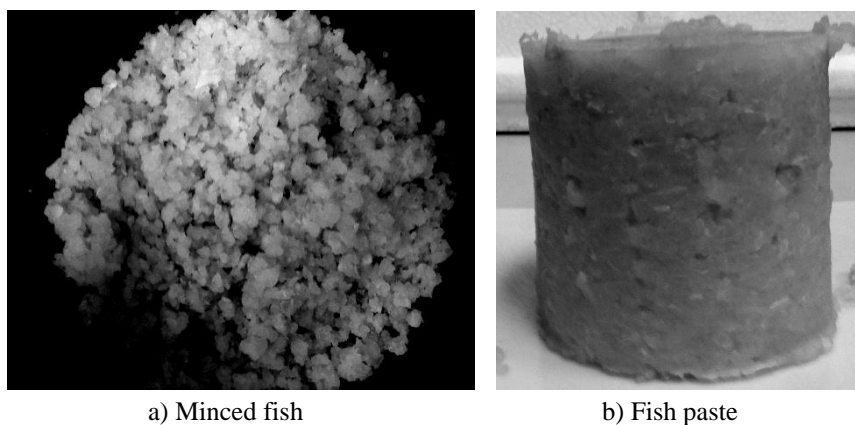
### **2.2 Preparation of snakehead fish frozen surimi**

Cultured snakehead fish (from 400 - 700 g) were purchased directly at the farming area in Tam Binh district, Vinh Long province. After collection, the fish was transported live (in a bucket of water) to the laboratory, which took about 1 hour. At the laboratory, live fishes were kept stable in the water tank for at least one hour before further processing.

Snakehead fishes were weighed prior to preliminary processing. Fishes were anesthetized, cut and discharged blood in the water tank (5 minutes to ensure complete separation of blood). Fishes

after exhaustion of blood were removed fins, skin, viscera, head and washed in 0.5% NaCl solution. After preliminary processing, fillet was taken to recover fish meat and washed with water at low temperature ( $5\div 10^{\circ}\text{C}$ ). Cut fish fillets ( $2\times 2\text{ cm}$ ) into PE bags (1 kg/bag) for freezing at  $-18\pm 2^{\circ}\text{C}$ . Fish meat must be stored frozen at least 24 hours before the study.

Frozen fish meat was cut evenly ( $<5\text{ mm}$ ) before washing to recover myofibrillar protein by a heated magnetic stirring. Perform one washing cycle with NaCl 0.15 M solution, 1:2 ratio of minced fish to solvent at  $35^{\circ}\text{C}$  and 14 minutes [9]. The wash mixture was compressed through a filter cloth to separate water (Fig. 1). The recovered fish paste was mixed with fixed amount of sugar, sorbitol, tripolyphosphate at 1.5%, 1.5%, 0.4% respectively to form surimi. The paste after mixing the additive was frozen and stored for at least 24 hours before conducting the study.



**Figure 1:** Minced fish and fish paste after dewatering

### 2.3 Fried fish cake preparation

Frozen fish surimi was roughly cut to break down the structure while simultaneously mixing the additives according to the study. Fixed amount of pepper, garlic powder and monosodium glutamate in the percentage of 0.5%, 0.5% and 0.3% was added respectively. Then the mixture was finely cut about 1 minute to create a paste before filling in petri dish to form the disk shape (diameter = 70 mm and thickness = 15 mm). The samples were kept cold in refrigerator at a temperature between  $2\text{-}4^{\circ}\text{C}$  to stabilize the gel structure before being pre-steamed at  $80\text{-}85^{\circ}\text{C}$  until the product central temperature rose to  $55^{\circ}\text{C}$ . The pre-steamed fish cake would be cooled and refrigerated from  $2\text{-}4^{\circ}\text{C}$  for at least 24 hours before deep frying at  $180^{\circ}\text{C}$  for 3 minutes (the ratio of frying oil and fish cake was 3:1). Analysis of fried fish cake parameters was carried out after keeping the sample in refrigerator for at least 24 hours.

In order to investigate the optimization of salt - water supplementation combined with other additives to improve the quality of snakehead fish cake, 3 experiments were prepared:

*Experiment 1:* Preparation of fried fish cake with different modified starches varying from 2%, 3% and 4%, control with no added modified starch. Other additives fixed in this experiment include 1.5% salt, no water and chitosan added.

*Experiment 2:* In preliminary experiments, the effect of each factor (water, salt) on fried fish cake processing was determined by the classical method (single-factor design) (unpublished data). It pointed out that salt and water strongly impacted on gel characteristics of fried fish cake. However, the values depended on the “one-variable-at-a-time” approach couldn’t explain the mutual interactions between the independent variables. Thus, in this study, the interactive effects of these two factors selected as key parameters were investigated for further optimization fried fish cake processing by response surface methodology approach basing on single-factor design optimization results. Response surface methodology with central composite design was applied to optimize the variables of the key factors (salt and water) and the effect of their interactions on fried fish cake with the purpose of obtaining the highest compressive strength ( $Y_1$  – CS,  $g_f$ ) and water holding capacity ( $Y_2$  – WHC, %). Central composite design was set up with 3 levels of each variable and 4 central points for the total of 12 treatments. The design was repeated twice with the center point as  $X_1 = 1$  (the ratio of water addition) and  $X_2 = 5$  (the ratio of salt addition). Other additives were fixed as the result of experiment 1.

*Experiment 3:* Perform fried fish cake processing with the level of addition of chitosan solution (dissolve in 1% solution of acid acetic with the volume equal the volum of water addition) was changed from 25, 50, 75 and 100 mg/100 g surimi, control with no added chitosan solution. Other additives were fixed as the result of experiment 2.

## **2.4 Analyses of proximate composition and physical properties**

The proximate composition analysis was determined according to AOAC methods [10]. Moisture content was determined by drying samples at 105°C for 5 hours. Protein content was determined using the Kjeldahl method, and lipid content was determined by Soxhlet method. Muscle recovery efficiency was defined as the ratio of fish muscle yield obtained during the processing to the amount before processing multiplied by 100.

The water holding capacity (WHC) was performed according to filter paper pressed method [11].

Compressive strength (CS) was carried out using a Rheometer [12].

Whiteness ( $WI = L^* - 3b^*$ ) was measured using Colorimeter NH300 [13].

## **2.5 Data analysis**

Using Statgraphics Centurion 16.1 program, data were analyzed for the degree of variation and significance of difference based on the analysis of variance (ANOVA) and LSD or Duncan test to determine if significant differences ( $p \leq 0.05$ ) existed between treatments. Optimization of the level of addition of salt and water by Using Response Surface Methodology with central composite design.



### 3 RESULTS AND DISCUSSION

#### 3.1 Physical and chemical properties of snakehead fish muscle

The results of chemical and physical analysis of cultured snakehead fish muscle (fillet, paste) were summarized in Table 1.

The results in Table 1 showed that snakehead fish fillet had a moisture content of 77.55%, protein content of 18.67%, and lipid content of 2.46% - similar to that of Kulachi et al. [14] on cultured snakehead fish in Multan (Pakistan) - with moisture content from 77.00 to 78.92%, protein content from 15.75 to 17.50% and fat content from 2.25 to 2.50%. According to a study by Firlianty et al. [15], in the Channidae family, *Channa striata* has the best meat quality with protein content of 20.83%, lipid content of 0.49%, and moisture content of 77.64%.

**Table 1:** Chemical and physical properties of cultured snakehead fish muscle; different letters in the same rows indicate significant differences in the test treatments at 95% confidence intervals; \* Calculated according to the amount of fish fillet; values are given as means  $\pm$  stdev

Proximate parameters	Fillet	Paste
Moisture (%)	77.55 <sup>a</sup> $\pm$ 0.31	78.37 <sup>b</sup> $\pm$ 0.39
Protein (%)	18.67 <sup>a</sup> $\pm$ 0.39	16.45 <sup>b</sup> $\pm$ 0.26
Lipid (%)	2.46 <sup>b</sup> $\pm$ 0.09	2.07 <sup>a</sup> $\pm$ 0.10
WHC (%)	68.27 <sup>a</sup> $\pm$ 0.45	71.70 <sup>b</sup> $\pm$ 0.51
pH	6.72 <sup>a</sup> $\pm$ 0.12	6.88 <sup>a</sup> $\pm$ 0.16
Muscle recovery efficiency (%)	46.84 $\pm$ 1.14	72.16 $\pm$ 1.24*

Together with the high muscle recovery efficiency (46.84%), the results showed the great promise of using snakehead fish in the Mekong Delta in general and Vinh Long Province in particular in processing products with protein characteristics. The results in Table 1 also showed that the water holding capacity of snakehead fish fillet was 68.27%, indicating that the quality of snakehead fish fillet was good. In terms of moisture and water retention capacity, snakehead fish fillets had comparable characteristics compared to fresh fish muscle, 78.31% and 68.45%, respectively [16].

The results also showed that washing increased water holding capacity of fish muscle compared to raw fish fillet and reached 71.70%. This may be due to washing process that removed soluble protein and other components from the fish muscle, increasing the muscle myofibrillar protein concentration in the paste (total protein up to 19.45%) [17]. High water holding capacity of myofibrillar protein also led to an increase in moisture content of the paste (78.37%). The removal of the sarcoplasmic protein, lipid, pigments as well as the pH of fish paste was



maintained at high pH (pH 6.88) by washing with 0.15 M NaCl solution (pH10) which improved gel formation of fish paste [18]. In addition, the fluctuation of the raw material index was not much (less than 2%) showed the uniformity of the material sources have been guaranteed.

All of these had shown that snakehead fish paste after washing was suitable for processing into protein-based products (fish cake) but also required appropriate solutions to enhance the gel properties of the product. In this study, the use of food additives to improve the quality of fish cake has been implemented.

### **3.2 The effect of modified starch supplementation on the quality of fried fish cake**

Starch was commonly used in protein gel products due to their ability to co-gel with protein by hydrogen bonds and Van der Waals [19]. The appropriate starch supplementation improved gel characteristic of fish cake, making this product more attractive for its properties. The effect of modified starch on the change in water holding capacity and compressive strength of fried fish cake was summarized in Table 2.

**Table 2:** The effect of supplementation of modified starch on water holding capacity (WHC) and compressive strength (CS) of fried fish cake; different letters in the same columns indicate significant differences in the test treatments at 95% confidence intervals; values are given as means  $\pm$  stdev

Modified starch (%)	WHC (%)	CS (gf)
0	87.43 <sup>a</sup> $\pm$ 0.31	484.04 <sup>a</sup> $\pm$ 8.16
2	89.54 <sup>b</sup> $\pm$ 0.39	507.70 <sup>b</sup> $\pm$ 9.11
3	91.34 <sup>d</sup> $\pm$ 0.42	523.70 <sup>c</sup> $\pm$ 7.64
4	90.54 <sup>c</sup> $\pm$ 0.36	517.37 <sup>bc</sup> $\pm$ 7.65

The results in Table 2 confirmed that the supplementation of modified starches had a great impact on gel characteristics of fried fish cake. The water holding capacity and compressive strength of the product significantly improved by an increase of the percentages of modified starch from 0% to 3%. However, higher levels of modified starch supplementation did not improve gel properties. In addition, Cierach et al. [7] also showed that the improvement of the water holding capacity of the modified starch was due to pH improvement of modified starch.

The results also demonstrated that although modified starch played an active role in protein gel products due to its gelation phenomenon, the use of starch in these products was limited. The high modified starch content (4%) would negatively affect gel characteristics of product [6]. The presence of modified starch at high level affected the co-gelation of protein with modified starch, which is the cause of the reduction of gel properties in the product. As a result, the water holding capacity and compressive strength of the fish cake had decreased.

From the above results, it can be concluded that the addition of 3% modified starch significantly improved the gel characteristics of the fried fish cake made from snakehead fish surimi with the highest of water holding capacity and compressive strength.

### 3.3 Optimize level of addition of water and salt in fried fish cake processing

Based on the central composite design analysis, the final model was obtained by multiple regression analysis of the experimental data of compressive strength and expressed by the following equation 1

$$Y_1 = 361.09 + 16.53X_1 + 275.56X_2 - 3.09X_1^2 + 10.90X_1X_2 - 122.33X_2^2 \quad (1)$$

Where,  $Y_1$  (gf) was the predicted compressive strength of fried fish cake;  $X_1$  (%) and  $X_2$  (%) are the coded values of water and salt addition, respectively.

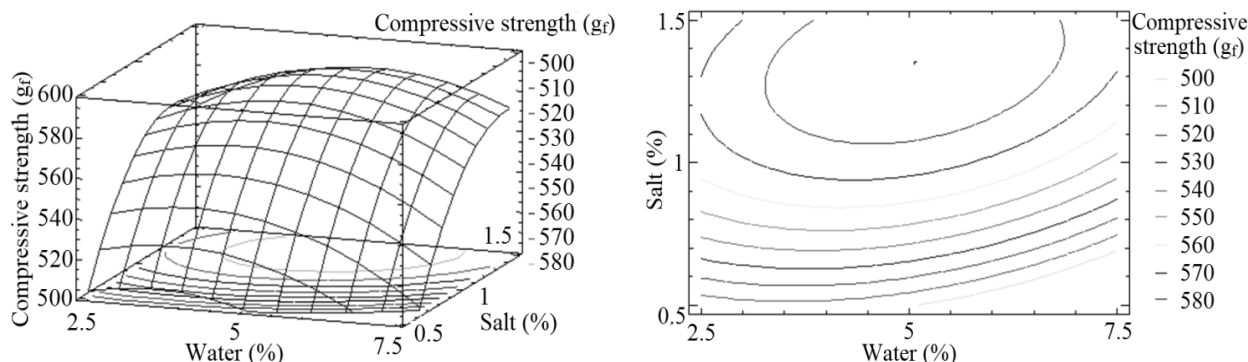
To validate the statistical results and the model equation, an analysis of variance (ANOVA) was conducted and the results are shown in Table 3. The high F-values and the values of P less than 0.05 implied significant model fit. The "Lack of Fit F-value" of 0.09 implies the Lack of Fit is not significant relative to the pure error. There is a 9% chance that a "Lack of Fit F-value" this large could occur due to noise. Thus, the lack of fit is insignificant. It meant the model was good. The high value of regression coefficient (R-squared = 97.28%) and the adjusted R-squared of 96.82%. These suggested that the regression model was an accurate representation of the experimental data. These findings indicated that the model equation obtained was appropriate for predicting the effects of water and salt addition on compressive strength of fried fish cake. It can also be seen from Table 2 that the linear and quadratic effects of water and salt addition, as well as the interactive effect between water and salt addition were highly significant ( $P < 0.05$ ). The optimum level of each variable and the effect of their interactions on compressive strength of fried fish cake were studied by plotting three dimensional response surfaces (Fig. 2).

**Table 3:** ANOVA for compressive strength of fried fish cake

Factors	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value
$X_1$ : % water	1773.01	1	1773.01	49.76	0.00
$X_2$ : % salt	43753.90	1	43753.90	1227.94	0.00
$X_1X_1$	7145.65	1	7145.65	200.54	0.00
$X_1X_2$	2227.69	1	2227.69	62.52	0.00
$X_2X_2$	17958.60	1	17958.60	504.00	0.00
Blocks	24.54	2	12.27	0.34	0.72

Factors	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value
Lack-of-fit	1617.94	19	85.15	2.39	0.09
Pure error	320.69	9	35.63		
Total (corr.)	71147.70	35			

R-squared = 97.28 percent; R-squared (adjusted for d.f.) = 96.82 percent



**Figure 2:** Three-dimensional response plots showing interaction effects of water and salt addition on compressive strength

The three-dimensional curves of the calculated responses show the interactions between water and salt addition (Fig. 2). From Fig. 2, it can be revealed that both additives have significant influence on the compressive strength. To further validate optimal values, the optimal values of the variables affected the compressive force of fried fish cake given by the software which calculated the equation giving the following results:  $X_1 = 5.07$ ;  $X_2 = 1.35$ . Therefore, the optimal values of the variables combination were the following: water addition was 5.07%, salt addition was 1.35%. According to the results of the statistically designed experiments, the fried fish cake processing was performed under this optimal condition for maximum compressive strength. The maximum predicted value of compressive strength was 589.21 gf, more than previous optimization result by “one-variable-at-a-time” method (540.37 gf) (unpublished data). Therefore, the response surface optimization could be successfully used to determine the level of addition of water and salt and to maximize the compressive force in this study.

Similar to compressive strength, the final model was obtained by multiple regression analysis of the experimental data of water holding capacity and expressed by the following equation 2.

$$Y_2 = 84.36 + 1.32X_1 + 6.40X_2 - 0.19X_1^2 + 0.26X_1X_2 - 3.2X_2^2 \quad (2)$$

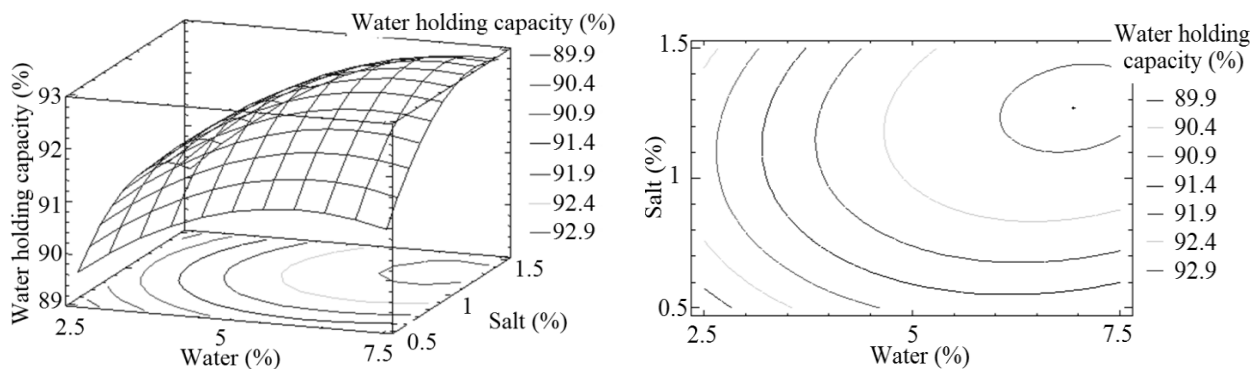
Where,  $Y_2$  (%) was the predicted water holding capacity of fried fish cake;  $X_1$  (%) and  $X_2$  (%) are the coded values of water and salt addition, respectively. The analysis of variance (ANOVA) was conducted and the results are shown in Table 4.

**Table 4:** ANOVA for water holding capacity of fried fish cake

Factors	Sum of Squares	Degree of freedom	Mean Square	F-value	P-value
X <sub>1</sub> : % water	22.81	1	22.81	1126.66	0.00
X <sub>2</sub> : % salt	9.18	1	9.18	453.54	0.00
X <sub>1</sub> X <sub>1</sub>	10.53	1	10.53	520.01	0.00
X <sub>1</sub> X <sub>2</sub>	1.25	1	1.25	61.98	0.00
X <sub>2</sub> X <sub>2</sub>	12.51	1	12.51	617.86	0.00
Blocks	0.02	2	0.01	0.52	0.61
Lack-of-fit	0.72	19	0.04	1.88	0.17
Pure error	0.18	9	0.02		
Total (corr.)	53.38	35			

R-squared = 98.30 percent; R-squared (adjusted for d.f.) = 98.02 percent

With high value of regression coefficient (R-squared = 98.30%), adjusted R-squared of 98.02% and the parameters in Table 4 shown the suitability of the regression equation as well as the effect implications of the factors on the objective function Y<sub>2</sub> (water holding capacity) was statistically significant. As for compressive strength, the water holding capacity of fried fish cake was greatly influenced by the change in water and salt addition.

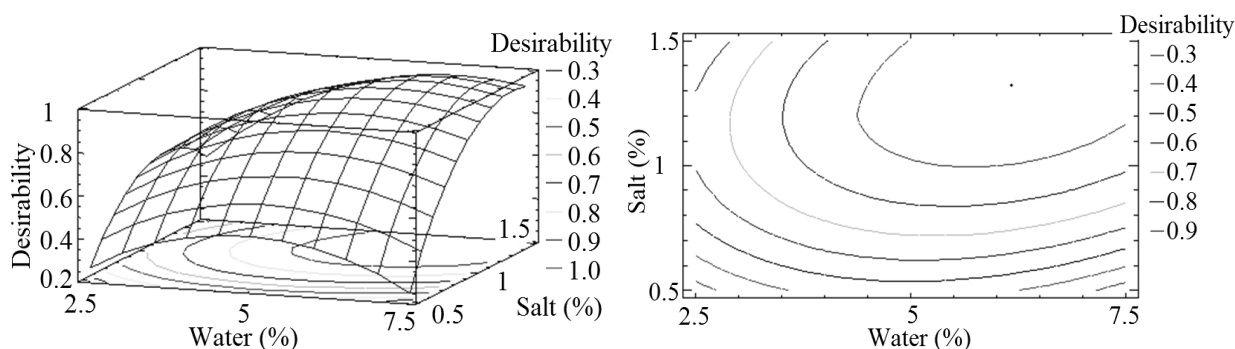


**Figure 3:** Three-dimensional response plots showing interaction effects of water and salt addition on water holding capacity

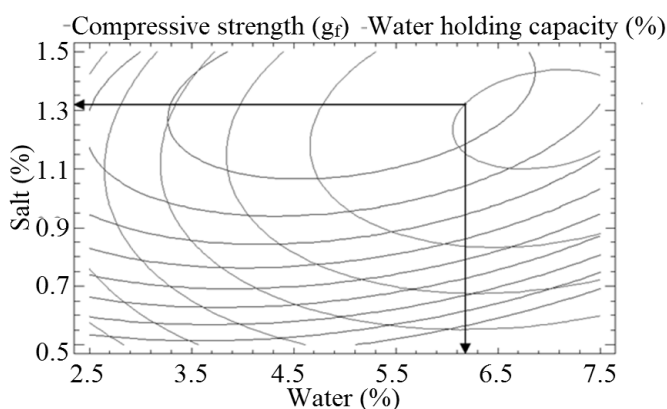
Fig. 3 also demonstrated the effect of water and salt addition on water holding capacity of fried fish cake. However, the optimum level of addition had an adjustment compared to the optimum level of addition for compressive strength. Specifically, to maximize water holding capacity, the

supplemental water must be increased to 6.94% and the rate of added salt must be reduced to 1.27%. Under optimal conditions, the water holding capacity of fried fish cake was 92.99%.

In order to determine the appropriate amount of water and salt addition to achieve the optimal gel characteristics of fried fish cake (max both compressive strength and water holding capacity), the optimization of multiple responses was carried out. As the compressive strength and water holding capacity depended on water and salt addition, the desired gel characteristic value of fried fish cake also varied according to the change of supplement rate of these additives (Fig. 4, Fig. 5).



**Figure 4:** Three-dimensional response plots showing interaction effects of water and salt addition on desired gel characteristic



**Figure 5:** Overlay plot showing interaction effects of water and salt addition on desired gel characteristic

Optimization of multiple responses showed that optimal water and salt addition to fried fish cake were 6.17% and 1.32%, respectively. At this optimal condition, the water holding capacity and compressive strength of fried fish cake reached 92.90% of 584.94 g<sub>f</sub>, respectively.

### 3.4 The role of supplemental chitosan solution in fried fish cake processing

In the presence of chitosan, the protein-polysaccharide complexes were formed between the amino group of glucosamine and the glutamyl group of myofibrillar proteins. These complexes had a great influence on gel characteristics in protein-based product [20, 21]. Thus, in fish cake, chitosan played a very important role in improving compressive strength, product stability, water

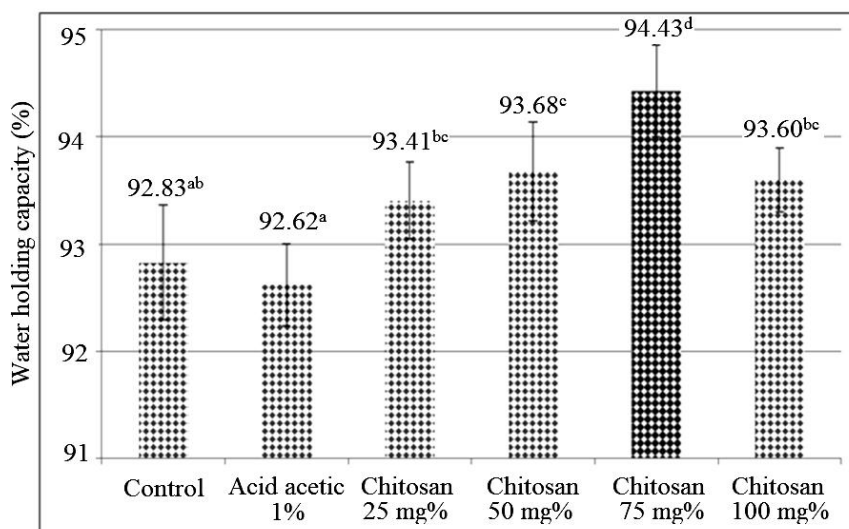
holding capacity and reduced microbial activity. Changing the ratio of chitosan would change the gel characteristic of fried fish cake. In addition, the amount of acetic acid used to dissolve chitosan was also a factor that could affect the gel properties of the product.

**Table 5:** Effects of supplementation of chitosan solution on gel and microorganism characteristics of snakehead fish cake; Different letters in the same columns indicate significant differences in the test treatments at 95% confidence intervals; values are given as means  $\pm$  stdev

Treatment	pH	Whiteness	Compressive strength (gf)	Total aerobic bacteria (cfu/g)
Control	7.03 <sup>b</sup> $\pm$ 0.08	72.11 <sup>ab</sup> $\pm$ 0.65	577.87 <sup>a</sup> $\pm$ 8.20	3.0 $\times$ 10 <sup>2</sup>
Acid acetic 1%	6.72 <sup>a</sup> $\pm$ 0.09	73.44 <sup>bc</sup> $\pm$ 0.65	569.73 <sup>a</sup> $\pm$ 7.95	7.5 $\times$ 10 <sup>1</sup>
Chitosan 25 mg%	6.75 <sup>a</sup> $\pm$ 0.08	73.99 <sup>c</sup> $\pm$ 0.53	658.08 <sup>b</sup> $\pm$ 8.75	9.0 $\times$ 10 <sup>1</sup>
Chitosan 50 mg%	6.85 <sup>a</sup> $\pm$ 0.11	73.78 <sup>c</sup> $\pm$ 1.01	687.89 <sup>cd</sup> $\pm$ 8.06	1.0 $\times$ 10 <sup>1</sup>
Chitosan 75 mg%	6.83 <sup>a</sup> $\pm$ 0.09	73.19 <sup>bc</sup> $\pm$ 0.75	702.61 <sup>d</sup> $\pm$ 9.79	0
Chitosan 100 mg%	6.83 <sup>a</sup> $\pm$ 0.09	71.79 <sup>a</sup> $\pm$ 0.70	676.63 <sup>c</sup> $\pm$ 9.30	0

However, Table 5 showed that the acetic acid solution did not significantly affect the pH of the product. Ishizaki et al. [22] suggested that the surface modification of actomyosin was very sensitive to pH and the pH range of 6.5 to 7.0 was considered suitable for gel formation of fish paste. Moreover, according to research by Dey and Dora [23], the addition of chitosan would be very effective in improving the ability of water holding capacity of fish meat.

Results in Table 5 showed the effects of different chitosan additions on the gel characteristics of fried fish cake. Increasing the chitosan level of addition to 75 mg/100 g of surimi improved the water holding capacity and compressive strength of the product. However, when the chitosan level of addition increased to 100 mg/100 g surimi, these characteristics tended to decrease (Fig. 6). This can be explained by the fact that even though chitosan can bind to muscle protein, but with chitosan excess, the interaction between protein and chitosan in the gel system decreases, which in turn results in poorer gel characteristics.



**Figure 6:** The effect of different chitosan ratios on water holding capacity of fried fish cake; values are given as mean  $\pm$  stdev

A study by Kungsuwan et al. [8] on surimi produced from Thai catfish (*Pangasius sutchi*) showed that with 30 mg chitosan/100 g surimi resulted in improved gel characteristics of the product.

The study by Kataoka et al. [21] also demonstrated that the structure of the Walleye pollock surimi product could be doubled by the addition of 1.5% chitosan due to the acceleration of myosin heavy chain polymerization in the presence of chitosan, , whereas the addition of chitin to the surimi did not affect gel formation. In addition, according to research by Anwar et al. [23], chitosan supplementation would incorporate cryoprotectant in the product for positive effect in improving the quality of milk fish.

The results have also shown that the addition of chitosan influenced the color of the product when the chitosan level of addition is high (from 100 mg/100 g surimi). When the chitosan level of addition increased from 25 to 75 mg/100 g surrими, the whiteness of the product was not altered but was whiter than that of control sample. This was mainly due to the effect of 1% acetic acid used to dissolve chitosan, whereby acetic acid reduced the pH near the pI value of fish muscle (isoelectric point), resulting in a decreased in negative charges in the meat, concurrently myoglobin in meat was also oxidized under the action of acid. According to Chow et al. [24], adjusting the pH of the paste to pI will increase the whiteness of "kamaboko" by causing denaturation and oxidation of myoglobin in meat. The study by Amiza and Kang [25] also demonstrated that the addition of suitable level of chitosan resulted in a significant improvement in gel whiteness from African catfish surimi, but excess amount of chitosan would lead to decrease in whiteness.

Improving the color of the product might also be due to the rearrangement and interaction of water molecules, proteins and polysaccharides when chitosan was added. The interaction between



chitosan-chitosan and the chitosan-protein complex changed the gel network, which indirectly improves the whiteness of the product [26]. Besides, results in Table 6 also showed that the addition of chitosan played a vital role in controlling the growth of microorganisms. This was evident by the addition of chitosan from 75 mg/100 g surimi.

Results of the study have demonstrated the role of chitosan supplementation to improve gel properties and microbiological safety of fried fish cake. Based on the results of chitosan investigations, the ratio of chitosan supplemented from 75 mg/100 g of surimi has improved the gel properties of the product.

#### 4 CONCLUSIONS

From the results of the research, the role of some additives in the quality of fried fish cake has been identified as follows:

- The addition of 3% modified starch improved the water holding capacity and compressive strength of the fried fish cake made from snakehead fish surimi.
- A correlation model between water and salt addition was established for water holding capacity and compressive strength of fried fish cake made from snakehead fish surimi with relatively high correlation coefficient, the optimal of water and salt addition was 6.17% and 1.32% respectively. Under optimum condition, the water holding capacity and compressive strength of the product reached 92.90% and 584.94 g<sub>f</sub>, respectively.
- The supplementation rate of chitosan 75 mg/100 g surimi was suitable to improve the gel characteristics of fried fish cake.

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## OPTIMIZATION OF EXTRACTION CONDITION OF ESSENTIAL OIL FROM *CITRUS LIMONIA OSBECK* PEEL BY RESPONSE SURFACE METHOD

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### ABSTRACT

The process of enzyme- vacuum-assisted distillation for extraction of peel essential oil from *Citrus limonia Osbeck* was optimized using response surface methodology (RSM). The factors considered were material to water ratio (A), incubation time (B) and concentration of pectinase (C). These parameters were varied at two levels. The material to water ratio of 0.39 g/ml, incubation time of 60.69 min and pectinase concentration of 296.99 U/ml were predicted as the optimum conditions. These factors gave an optimum oil yield of 0.978%. Analysis of variance (ANOVA) indicated that the model was significant as evidenced from  $R^2$  of 0.9929 and the model F-value of 92.86. The experimental value (0.975 % oil yield) was close to the predicted value (0.978%). Therefore, the model could be used for prediction of oil yield in essential oil extraction from *Citrus limonia Osbeck* peel using enzyme- and vacuum-assisted distillation method.

**Keywords:** Optimization, response surface methodology, distillation, essential oil, Citrus.

### 1 INTRODUCTION

*Citrus* is a common term and genus of flowering plants that belongs to the *rue* family, *Rutaceae*, originating and growing extensively in tropical and subtropical southern regions of Asia. *Citrus* is an important agricultural crop world-wide. In 2010, the production of *citrus* fruit worldwide was estimated as 122.5 million tones and oranges accounted for 50%–62% of the total area harvested and total production [1]. *Citrus* fruits' essential oils called agrumen oils that are considered generally recognized as safe (GRAS) [2]. The essential oils of *citrus* can be extracted from flavedo of *citrus* fruits. The essential oils from peels are a mixture of volatile compound and mainly consisted of monoterpenes hydrocarbons [3]. In addition, the *citrus* peel oils comprise over a hundred other constituents that can be divided into two fractions: sesquiterpene hydrocarbons and oxygenated compounds [3,4].

*Citrus* peel essential oils were usually extracted by cold pressing or hydro-distillation method [5]. In cold pressing method, the smell of essential oil is natural, chemical composition is conservative. Nevertheless, the yield of essential oils extracted using this process is low. In contrast, the essential oils extracted by hydro-distillation have wide acceptance for large scale production because of high yield. The drawback of this process is that the hydrolysable compounds such as ester, as well as thermally labile components, may be decomposed during the distillation process [6]. This has driven researcher and manufacturers to search for new methods of extraction in order to improve the yield without alteration of the qualitative traits of the product extracted. An alternative for essential oil production using enzymatic pre-treatment of vacuum-distillation is necessary. Vacuum distillation used in the study was modified from hydro-distillation method to reduce the boiling point and avoid altering volatile compounds while application of enzyme is to hydrolyze partial or complete of the cell walls and increase of oil yield. In the extraction by enzyme-assisted vacuum distillation, the extraction parameters need to be optimized.

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for modeling and analyzing of problems in which a response of interest is influenced by some quantitative variables with the objective of optimizing the response. A number of studies used RSM to optimize essential oils extraction processes. Extraction process of dried *Patchouli* leaves essential oil and *Eucalyptus tereticornis* essential oil was optimized by RSM [7,8].

The aim of this study is to optimize the conditions of enzyme-assisted vacuum distillation of *citrus* peel essential oil (*Citrus limonia Osbeck*) using Response Surface methodology. The process parameters of (1) material to water, (2) incubation time, and (3) concentration of pectinase were treated as independent variables in the three-level three-factorial Box-Behnken design.

## 2 MATERIALS AND METHODS

### 2.1 Materials

*Citrus limonia Osbeck* were obtained from Da Lat province, Vietnam. The fruits albedo layers were peeled off carefully and discarded while the flavedo were kept for extracting the essential oils.

### 2.2 Extraction method of essential oil

Essential oils of *citrus* peels were extracted using a modified Clevenger-type apparatus under vacuum condition. The maintenance pressure of the system was controlled at 575mm Hg. Before extraction, *citrus* peels were subjected to a preliminary treatment by soaking in water, then put into a 2 L glass beaker equipped with a hot plate and a stirrer. After adjusting the medium to a pH value of 4.5, pectinase enzyme was added. Incubation was then performed under stirring at 45°C

for 30-90 min. Then, the material was vacuum distilled in a Clevenger-type apparatus for specified times (3 hours). The obtained essential oil was dried over anhydrous sodium sulfate and stored in a sealed vial at 4°C in the dark prior to analysis. Oil yield is calculated based on the mass of essential oil obtained and the mass of the initial material as following equation:

$$\text{Yield(\%)} = \frac{\text{weight of extracts recovered}}{\text{Weigh of fresh leaf}} \times 100 \quad (\text{Equation 1})$$

### 2.3 Box-Behnken design

Response surface methodology, in particular the Box-Behnken design was employed to estimate the effect of 3 reaction parameters namely material to water ratio (A), incubation time (B) and pectinase concentration (C) on yield of essential oil. These variables each at two levels, low and high: A (0.25-0.5 g/ml), B (30-90 min) and C (150-450 U/ml) are presented in Table 1. These levels were chosen based on the capacity of the experimental set up for variables A and B, while C was selected based on experimental run time. The experimental design is shown in Table 2 [9].

**Table 1:** Design summary

Factor	Name	Units	Low actual	High actual	Low code	High code
A	Material to water ratio	g/ml	0.25	0.5	-1	1
B	Incubation time	Min	30	90	-1	1
C	Pectinase concentration	U/ml	150	450	-1	1

**Table 2:** Box-behnken matrix

Run	A	B	C	Yield %
1	0.5	30	300	0.26
2	0.25	30	300	0.37
3	0.5	90	300	0.53
4	0.25	90	300	0.10
5	0.375	30	150	0.42
6	0.375	90	150	0.37
7	0.375	30	450	0.37
8	0.375	90	450	0.42
9	0.5	60	150	0.47
10	0.25	60	150	0.38

Run	A	B	C	Yield %
11	0.5	60	450	0.46
12	0.25	60	450	0.29
13	0.375	60	300	0.95
14	0.375	60	300	0.92
15	0.375	60	300	1.00
16	0.375	60	300	1.03

The regression analysis was performed to estimate the response function as a second order polynomial:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k \beta_{ij} X_i X_j \quad (\text{Equation 2})$$

where Y is the predicted response,  $X_i$  is the uncoded value of the i-th test variable,  $\beta_0$ ,  $\beta_i$  and  $\beta_{ij}$  are coefficients estimated from the regression according to Rajasimman et al. [7]. A statistical program package Design-Expert version 11 (State-Ease Inc., Minneapolis, MN, USA) was used for regression analysis of the data and for estimating the coefficient of the regression equation. The equations were validated by the analysis of variance (ANOVA) test. Model and regression coefficients were considered significant when the p-value was lower than 0.05.

### 3 RESULTS AND DISCUSSION

#### 3.1 Box-Behnken design analysis

Analysis of variance for the response surface model is presented in Table 3. The analysis indicates that the model F-value of 92.86 implies the model was significant. There was only a 0.01% chance that an F-value could occur due to noise, the model also has a satisfactory level of adequacy ( $R^2$ ).

**Table 3:** Analysis of variance (ANOVA) for response surface quadratic model to identify significant factors affecting the *citrus* oil yield

Source	Sum of Squares	df	Mean Square	F-value	p-value
<b>Model</b>	1.25	9	0.1385	92.86	< 0.0001
A-ratio	0.0417	1	0.0417	27.94	0.0019*
B-time	3.125E-06	1	3.125E-06	0.0021	0.9650
C-concentration	0.0013	1	0.0013	0.8803	0.3843
AB	0.0722	1	0.0722	48.41	0.0004*

Source	Sum of Squares	df	Mean Square	F-value	p-value
AC	0.0016	1	0.0016	1.07	0.3403
BC	0.0026	1	0.0026	1.76	0.2328
A <sup>2</sup>	0.4340	1	0.4340	290.87	< 0.0001*
B <sup>2</sup>	0.4422	1	0.4422	296.41	< 0.0001*
C <sup>2</sup>	0.2513	1	0.2513	168.41	< 0.0001*
<b>Residual</b>	0.0090	6	0.0015		
Lack of Fit	0.0017	3	0.0006	0.2262	0.8732
Pure Error	0.0073	3	0.0024		
<b>Cor Total</b>	1.26	15			
R <sup>2</sup>	0.9929				
Adj. R <sup>2</sup>	0.9822				
Pred. R <sup>2</sup>	0.9686				
Adeq. precision	28.419				

In this study, A, AB, A<sup>2</sup>, B<sup>2</sup> and C<sup>2</sup> were significant model terms ( $p < 0.05$ ). The lack of Fit F-value of 0.23 implied the lack of Fit was not significant relative to the pure error. There was 87.32% chance that a lack of Fit F-value could occur due to noise. The Predicted R<sup>2</sup> of 0.9686 was in reasonable agreement with the Adjusted R<sup>2</sup> of 0.9822; i.e. the difference was less than 0.2. Adeq Precision measured the signal to noise ratio (A ratio greater than 4 was desirable). The ratio of 28.419 indicated an adequate signal. This model can be used to navigate the design space.

### 3.2 Effect of process variables on the yield of essential oil

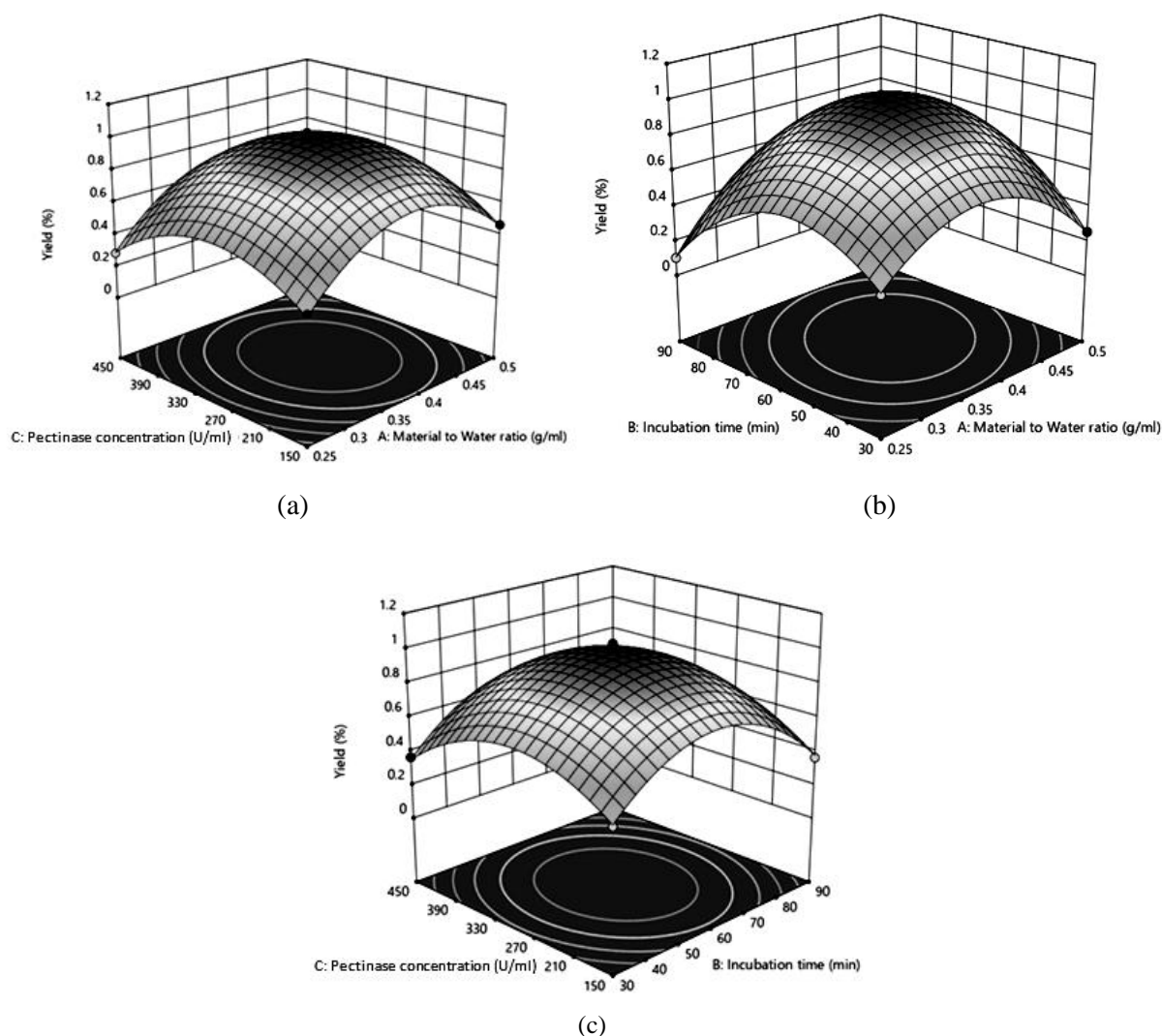
The response surface curves for extraction of *citrus* peel oil using enzyme-assisted vacuum-distillation plotted to understand the interaction of the variables and the optimum level of each variable for maximum response are presented in Fig. 1. Fig. 1a shows the interaction of material to water ratio and incubation time on the yield, while Fig. 1b and 1c indicates interaction of material to water ratio and pectinase concentration on the yield as well as that of incubation time and pectinase concentration on the yield, respectively.

Final equation in terms of actual variables is given by Equation 3.

$$\begin{aligned} \% \text{Yield} = & -3.5 + 13.9A + 0.03B + 5.8 \times 10^{-3}C + 0.03AB + 1.1 \times 10^{-3}AC + \\ & + 5.7 \times 10^{-6}BC - 21.1A^2 - 3.7 \times 10^{-4}B^2 - 1.1 \times 10^{-5}C^2 \end{aligned} \quad (\text{Equation 3})$$

Where A is material to water ratio, B is incubation time and C is pectinase concentration.





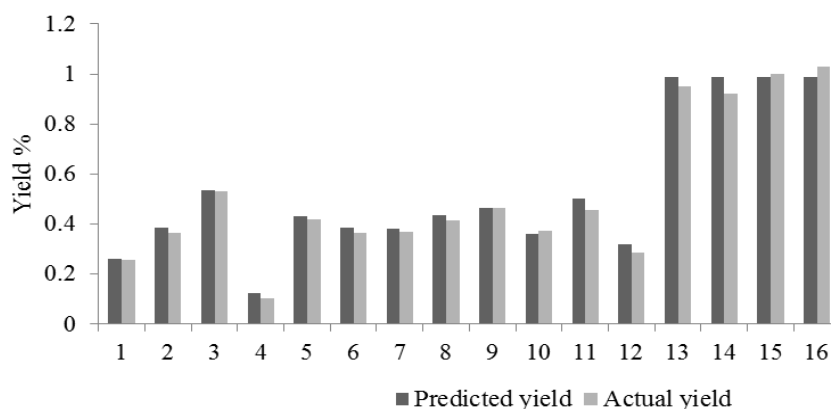
**Figure 1:** Response surface showing effect of (a) material to water ratio and incubation time on the yield at constant pectinase concentration (300 U/ml); (b) material to water ratio and pectinase concentration on the yield at constant incubation time (60 mins); (c) incubation time and pectinase concentration on the yield at material to water ratio (0.375 g/ml)

### 3.3 Yield of essential oil at different conditions

Experimental data generated in Table 2 in terms of actual values were substituted into Equation 3 and the predicted *citrus* peel oil yield was obtained. Actual *citrus* oil yield and the predicted yield are presented in Figure 2. Figure 2 indicates that the predicted values and actual values are in close agreement. This suggests good reliability of the model as also evidenced from the statistical parameters of the model such as standard deviation of 0.04,  $R^2$  of 0.9929 and F-value of 92.86. This shows fitness of the data for the model.

The optimum conditions for the response were predicted by numerical optimization method. The material to water ratio of 0.39 g/ml, incubation time 60.69 min and pectinase concentration of 296.99 U/ml were the optimum conditions for the yield. This condition gives a yield of 0.978%.





**Figure 2:** Predicted and actual oil yield from the model

## 4 CONCLUSIONS

The response surface methodology technique was applied to extract citrus peel essential oil by enzyme-assisted vacuum-distillation. The model design was significant. Material to water ratio, incubation time and concentration of pectinase were the major process parameters found to significantly influence the oil yield. The optimum conditions were 0.39 g/ml material to water ratio, 60.69 min incubation time and 296.99 U/ml of pectinase concentration giving 0.978 % of essential oil yield.

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## **SESSION 4**

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# **AGRICULTURE AND BIOTECHNOLOGY**



## THE EFFECT OF *SACCHAROMYCES SP. NO.9* ON PHYSICOCHEMICAL CHARACTERISTIC OF AGED ROSELLE WINE

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### ABSTRACT

Due to the color of Roselle calyxes, Roselle wine has been considered as one of the alternatives to regular red grape wine in the region where red grapes of wine quality is not easily found. The study of the effect of three yeast strains on sensory and chemical characteristics of Roselle wine by Lhamo (2015) showed that the Roselle wine aged with *Saccharomyces sp. No.9* had some distinct sensory characteristic as well as demonstrated more mouth feel than the other yeasts used for aging. Continuing in this research, the effects of different amount of *Saccharomyces sp. No.9* inoculum on physicochemical characteristic of aged Roselle wine was studied. The based wine was prepared by fermenting Roselle extract with Pasteur red yeast. The *Saccharomyces sp. No. 9* was added to base wine as aging inoculum with 2 different percentages (10% v/v and 20% v/v) after the based wine was racked. The results showed that Roselle wine aged with *Saccharomyces sp. No.9* had significantly higher in alcohol content, color enhancement and anthocyanin content, and also significantly lower in reducing sugar and hue compared to control after aging for 12 weeks ( $p < 0.05$ ). There was no significant difference in physicochemical characteristic between different percentages of *Saccharomyces sp. No.9*. The result provided information that *Saccharomyces sp. No.9* could sustain level of anthocyanin in the wine during 12 weeks of aging. The higher anthocyanin in the wine aged with *Saccharomyces sp. No.9* could be beneficial to the color of the wine as showed in the analysis of hue and color enhancement. Therefore, the application of *Saccharomyces sp. No.9* for wine aging could possibly improve characteristic of Roselle wine which will be beneficial to the industry.

**Keyword:** Roselle wine, Aging yeast, *Saccharomyces sp.*, anthocyanin

## 1 INTRODUCTION

Fruit wines have traditionally been popular in the wine homemade and in the area where the climate is not suitable for growing wine grapes, like in Thailand. Fruit wines can be made from many tropical fruits and subtropical fruits [33] and virtually any plant matters that provide color, flavor and aroma [18]. However, the tropical fruits do not commonly used to produce red wines because they have low content of extractable red pigments as opposed to red varietal grapes [27]. However, the tropical fruits and herbs with potential for producing red wine is also available, such as Roselle (*Hibiscus sabdariffa*).

Roselle is belonging to the Malvaceae family. This plant is an important annual crop grown successfully in tropical and sub-tropical climates as well as in Thailand. The commercially important part of the plant is the fleshy calyx or Roselle's outer leaves. The Roselle calyx is rich in anthocyanins that vary in color from pink to blue and violet [20], and contains a mixture of organic acids such as citric acid, hydroxycitric acid, hibiscus acid, malic acid, tartaric acids, oxalic acid, ascorbic acid, and succinic acid [3, 14, 32]. These characteristics suggest that Roselle could be one of the great choices as an alternative to red grape to produce red color in wine.

Color is one of the most appealing properties of a red wine, and the principal sources of red color in wines come from the anthocyanins or their further derivatives that are extracted or formed during the vinification process. During wine aging, the concentrations of monomeric and copigmented anthocyanins in red wines declines constantly. Decreasing in concentration of anthocyanins were due to the adsorption of anthocyanin by yeast [25, 38], the formation of pyranoanthocyanins (vitisin A and vitisin B) with yeast metabolites [26], precipitation with proteins [8], polysaccharides or condensed tannins [17, 19, 24, 33, 37, 41], and the progressive and irreversible formation of more complex and stable anthocyanin derived pigments, such as various pyranoanthocyanins, polymeric anthocyanins produced from condensation between anthocyanin and/or flavan - 3 - ols directly or mediated by aldehydes, as well as their further derivatives [31, 33].

In winemaking, yeasts play an important role in the fermentation of wines because they turn sugar in the fruit juices into ethanol and carbon dioxide [30]. Moreover, aroma and flavor of wine were also affected through yeasts by products from fermentation. Yeasts are not only affecting the wine during primary fermentation but also affecting wine quality during aging process. Aging is the method of storing wine in proper container. During aging, the wine quality can be improved that may cause by spontaneous chemical reactions or facilitate by microorganisms [14, 34]. The one technique of wine aging that has used microorganism to help in improving the quality of wine is called biological aging technique which is carried out by yeasts. These yeasts can be used to transform components in wine and create special sensory features to wine [29]. Biological aging technique has been applied regularly in sherry wines [9].

According to previous research, the research of Effect of Three Isolated Aging Yeast Strains on Sensory and Chemical Characteristics of Roselle Wine [22], found that the *Saccharomyces sp.* No.9 had affected on Roselle wine including higher alcohol content, lower acidity, and more mouthfeel than other strains after 3 months aging.

Therefore, this research was set up to further investigate the effects of *Saccharomyces sp.* No.9 on other chemical compositions of aged Roselle wine especially the phenolic compounds.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Dried Roselle calyces were obtained from a local market in Bangkok, Thailand. Pasteur Red wine yeast strain and *Saccharomyces sp.* No.9 strain were collected from Wanjaroen's yeast stock [40].

### **2.2 Pasteur Red starter culture preparation**

The starter culture was prepared from Roselle extracts made from boiling dried Roselle in water at the ratio of 1:25 (w/v) for 10 minutes. The total soluble solid (TSS) was adjusted to 15 °Brix by adding cane sugar and measuring by hand refractometer (RHB - 32ATC, China). 2 g/L of Di - ammonium phosphate (DAP) and 1 g/L of Calcium carbonate ( $\text{CaCO}_3$ ) was added to provide nitrogen source and adjusted pH of Roselle extract respectively. The final pH was measured by using pH meter (HANNA pH211 Microprocessor pH meter, HANNA Instruments, China). Then, the Pasteur Red wine yeast which grew on YM slant for 2 days was inoculated into the must. The starter culture was fermented at room temperature ( $30 \pm 2^\circ\text{C}$ ) until the cell number reach to approximately  $1.00 \times 10^{11}$  cells/mL then inoculated into the wine must. TSS was monitored to ensure fermentation activity.

### **2.3 *Saccharomyces sp.* No.9 starter culture preparation**

The *Saccharomyces sp.* No.9 strain was grown on YM slant at room temperature for 2 days. Then, it was inoculated into the filtered wine starter (from 2.2) which was filter using diatomite earth and filter paper Whatman No.1 (0.45  $\mu\text{m}$  pore size). The *Saccharomyces sp.* No.9 starter culture was left at room temperature for 7 days or until the cell number reach approximately  $2.00 \times 10^{10}$  cells/mL then inoculated into the based wine.

### **2.4 Based Roselle wine preparation**

Dried Roselle was boiled in water at the ratio of 1:15 (w/v) for 10 minutes. The TSS was adjusted to 20 - 22 °Brix by adding cane sugar then followed by the addition of 2 g/L of DAP and 1 g/L of  $\text{CaCO}_3$ . The final pH was measured by pH meter. Then 10% Pasteur Red starter culture (v/v) was added into the must to provide the concentration of yeast at approximately  $1.00 \times 10^{10}$  cells/mL of must. All treatments were fermented at room temperature ( $30 \pm 2^\circ\text{C}$ ) for 8 - 9 days. During

active fermentation, the pH, TSS and gravity (Triple Scale Hydrometer, Brew Tapper, USA) were checked every day. After fermentation was finished which indicated by the stable level of TSS measured by refractometer, all wine samples were filtered using diatomite earth and filter paper Whatman No.1 (0.45 µm pore size) and bottled for further aging process.

## 2.5 Aged Roselle wine preparation

After filtration, the based wine was filled into the standard wine bottles. Each batch of base wines was divided into three aliquots. One was kept as control aging without addition of aging yeasts. Another two aliquots were added with different percentages of aging yeast at 10% (v/v) ( $1.5 \times 10^{12}$  cells/bottle) and 20% (v/v) ( $3 \times 10^{12}$  cells/bottle) of *Saccharomyces sp.* No.9 starter culture. All wines were aged at 10°C for 3 months.

## 2.6 Physicochemical analysis

The physicochemical characteristics were analyzed before the wine samples were aged and then every 3 weeks during the aging period till the end of aging (3 months). The basic chemical compositions of wine were determined including pH, total titratable acidity (TTA), volatile acidity (VA), total soluble solid (TSS), reducing sugar, alcohol content, phenolic compounds, and wine color.

The pH was measured using pH meter. TTA was determined using Official method of analysis (AOAC 926.12, 1990), and expressed as citric acid (g/L). VA was estimated by determination of fixed acid then subtracted from TTA. The VA estimated as acetic acid (g/L). TSS was measured with a hand refractometer and hydrometer. The reducing sugar was determined using DNS assay [23]. The alcohol content was determined using the complete traditional Ebulliometer (160000 - Complete traditional Ebulliometer, Laboratories Dujardin - Salleron, France). For phenolic compounds including phenolics, total phenolics, anthocyanin contents, and total polymeric pigments were measured according to Modified Harbertson and Adams assay [35]. For color measurement, the color density and hue were determined by using spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments Ltd., China). The color density was traditionally the sum  $A_{420} + A_{520} + A_{620}$ . Hue was calculated as the ration  $A_{420}/A_{520}$  [11]. While the color enhancement was determined by the method of Boulton [5]. Absorbance was measured at 520 nm on a spectrophotometer.

## 2.7 Statistical analysis

The chemical analysis's data was analyzed by Completely Randomized Design (CRD) and Duncan test using SAS 9.4. The experiments were conducted in triplicate. All the tests were analyzed at 95% level of confidence.



### 3 RESULTS AND DISCUSSION

#### 3.1 Effect of *Saccharomyces sp.* No.9 on acidity during aging of Roselle wine

In this research, the effect of *Saccharomyces sp.* No.9 addition on the acidity of Roselle wine were monitored during 3 months aging. The results showed that the pH, total titratable acidity (TTA as citric acid), and volatile acidity (VA as acetic acid) of Roselle wine aged with and without *Saccharomyces sp.* No.9 were not significantly different ( $p > 0.05$ ) during the aging period. In based wine (week 0), the pH, TTA, and VA value of control wine were 3.37, 1.73 g/L and 1.09 g/L respectively. Whereas, at week 12, the pH value was increased to 3.47, while TTA and VA value were decreased to 1.37 g/L and 0.86 g/L respectively. The different percentages of aging yeast added did not resulted in different pH, TTA and VA values of Roselle wine during aging period.

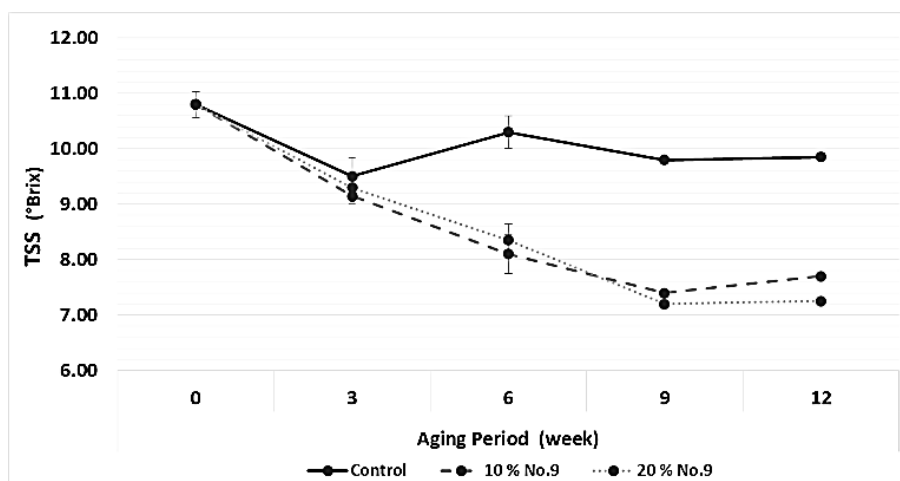
The pH of all treatments was in the range acceptable for standard commercial red wine (pH 3.3 - 3.6) [4]. While VA which usually representing contamination with acetic acid was also with in the range that acceptable for red wine ( $< 1.5$  g/L acetic acid) [39]. However, for TTA, the result cannot be compared directly with standard for red wine because the Roselle contains different types of acid. The Roselle calyx contains high percentages of organic acids, including citric acid (12 - 20%), hydroxycitric acid, hibiscus acid (13 - 24%), malic (2 - 9%) and tartaric acids (8%) as major compounds, and oxalic and ascorbic acid (0.02 - 0.05%) as minor compounds [32].

#### 3.2 Effect of *Saccharomyces sp.* No.9 on total soluble solid, reducing sugar and alcohol content

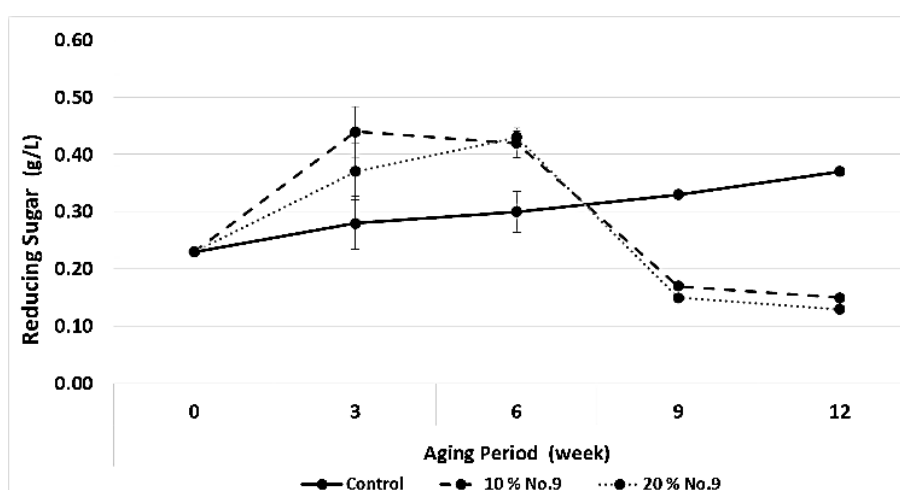
During aging period, the total soluble solids decreased with time (Figure 1), especially, the Roselle wines aged with *Saccharomyces sp.* No.9. The results in Figure 1 showed total soluble solids of Roselle wines aged with aging yeast were decreased more than control wine. This could be because of *Saccharomyces sp.* No.9 could converts remaining sugar in the wine to alcohol which was why the total soluble solids in wine were decreased during aging [4].

The result of reducing sugar (Figure 2) showed that the Roselle wine aged with aging yeast had increased in reducing sugar content in the first 6 weeks of aging compared to control (without aging yeast). After 6 weeks, the reducing sugar was decreased to 0.13 - 0.15 g/L while reducing sugar in control wine was not reduced. This result also correspond to the alcohol content was increased from 8.5% - 9.0% to 12.0% - 12.5% in the aged wine with *Saccharomyces sp.* No.9 (Figure 3). These results implied that some of the TSS components, e.g. oligosaccharides or undigested sucrose, may have been converted to fermentable sugars by *Saccharomyces sp.* No.9 during aging. These sugars were subsequently used by the yeast to produce more alcohol. While in control aged wine (without *Saccharomyces sp.* No.9), the changes in TTS, reducing sugar and alcohol contents were not as pronounce. However, the different percentages of aging

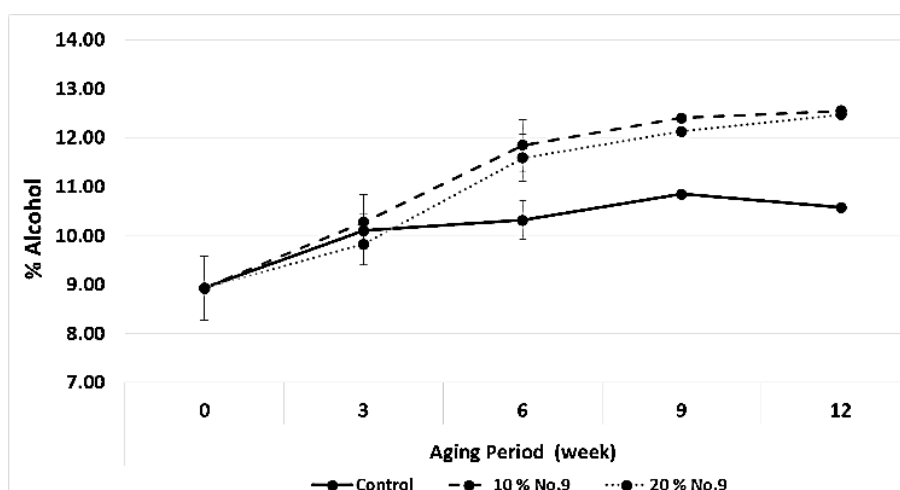
yeast added did not significantly affect total soluble solids, reducing sugar, and alcohol content of Roselle wine during aging period.



**Figure 1:** Total soluble solid during 3 months aging of Roselle wine



**Figure 2:** Reducing sugar during 3 months aging of Roselle wine



**Figure 3:** Alcohol content during 3 months aging of Roselle wine

### 3.3 Effect of *Saccharomyces sp.* No.9 on phenolic compounds and wine color

Color is one of the most appealing properties of a red wine, and the principal sources of red color in wines come from the anthocyanins. During wine aging, the concentration of anthocyanins in wine decreases progressively.

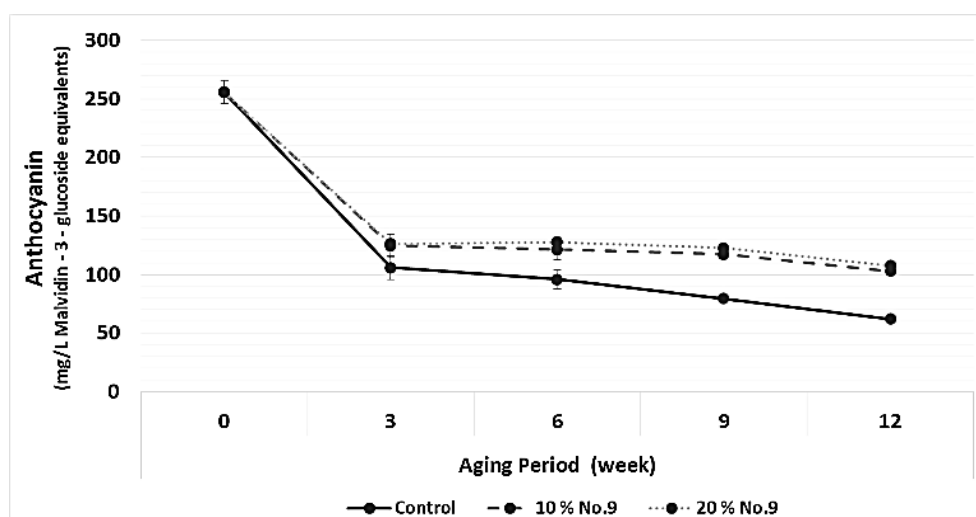
According to the level of anthocyanin content analyzed (Figure 4), the result showed that anthocyanins in all three treatments were shapely decreased during the first few weeks of aging. However, the wines aged with *Saccharomyces sp.* No.9 demonstrated slower reduction rate of anthocyanin than the control aged wine (without *Saccharomyces sp.* No.9) after the 3<sup>rd</sup> week of aging. This result might due to polymeric pigments formation by polymerization reaction between anthocyanin and another flavonoid substrate, such as flavan - 3 - ols (proanthocyanidins), or the formation of the 'bridge' mediated polymeric pigments from anthocyanins themselves or with flavan - 3 - ols [12]. As the result was observed in Figure 5, the amounts of polymeric pigments in three wine samples were slightly increased during aging period, however, the wines aged with *Saccharomyces sp.* No.9 had developed significantly lower amount of total polymeric pigments than the control during aging.

Decreasing in anthocyanin contents and increasing in polymeric pigment of aged wine during aging period have been demonstrated to have an effect on color of wine. More polymeric pigments formation could be the cause of changes in color where by the color shifts from red to orange and brick red [12]. The result of color enhancement and hue, in Figure 6 and Figure 7 respectively, showed a correlation between these two attributes. Theoretically, color enhancement represents red pigments in wine which was measured using spectrophotometer at wavelenght 520 nm, while hue is the ratio between yellow pigment (420 nm) and red pigment. When the absorption decreases at 520 nm and increases at 420 nm, the result representing shift of color of wine from red to an orange hue. The color enhancement of three samples was decreased during aging period, while the hue increased. However, the wine aged with *Saccharomyces sp.* No.9 showed significantly higher level of color enhancement and lower hue compared to the control aged wine.

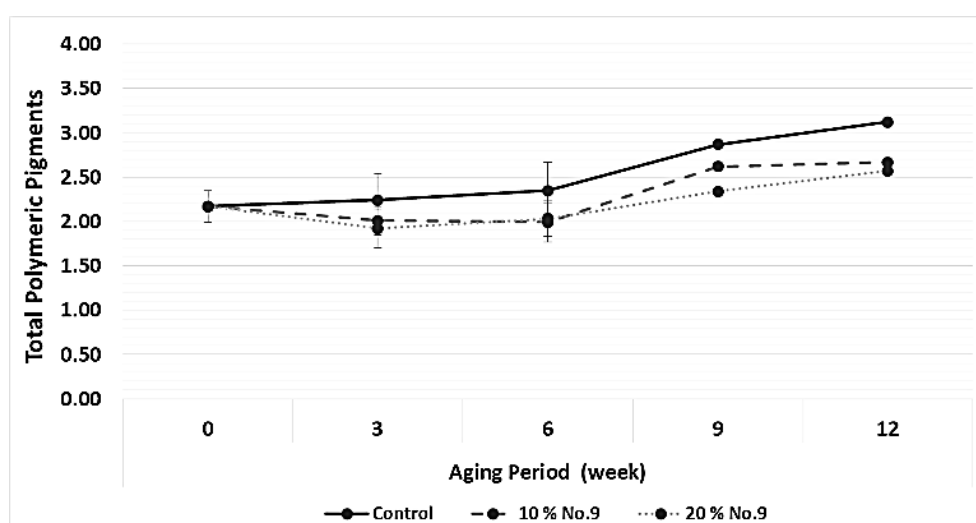
Results of color enhancement and hue were corresponded to level of anthocyanin which responsible for red, purple, and blue color. When the anthocyanin decreased during aging period, it also affected to color enhancement and hue. There were evidences indicated that the formation of pyranoanthocyanins between anthocyanin and yeast metabolites, e.g. pyruvate or acetaldehyde, could stabilized the color of wine during aging [26]. Therefore, the addition of *Saccharomyces sp.* No.9 may facilitate similar reactions which resulted in the stability of anthocyanin and color.

However, the different percentages of aging yeast did not affect anthocyanin content, total polymeric pigments, color enhancement, and hue of Roselle wine during aging period.

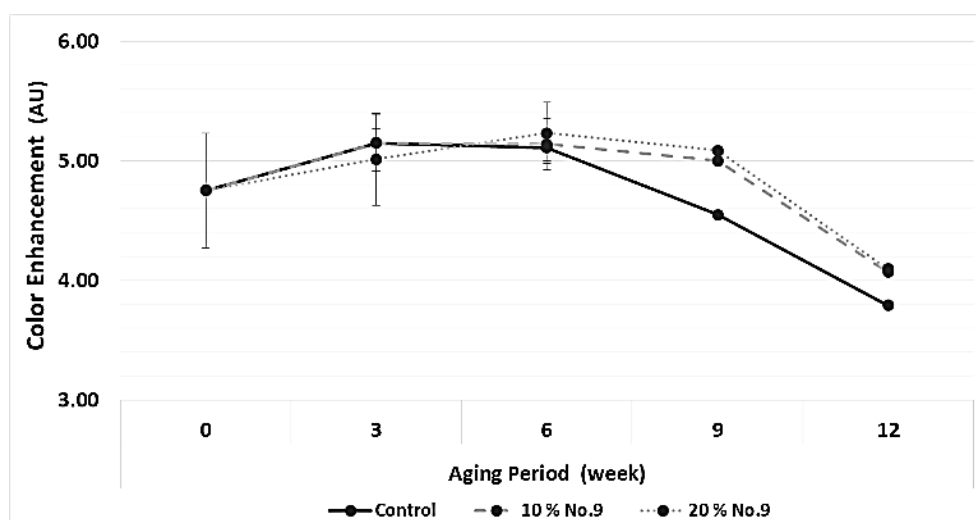
Apart from anthocyanina and total polymeric pigments, the analysis of total phenolic compound showed reduction with no significantly different between all treatments ( $p > 0.05$ ).



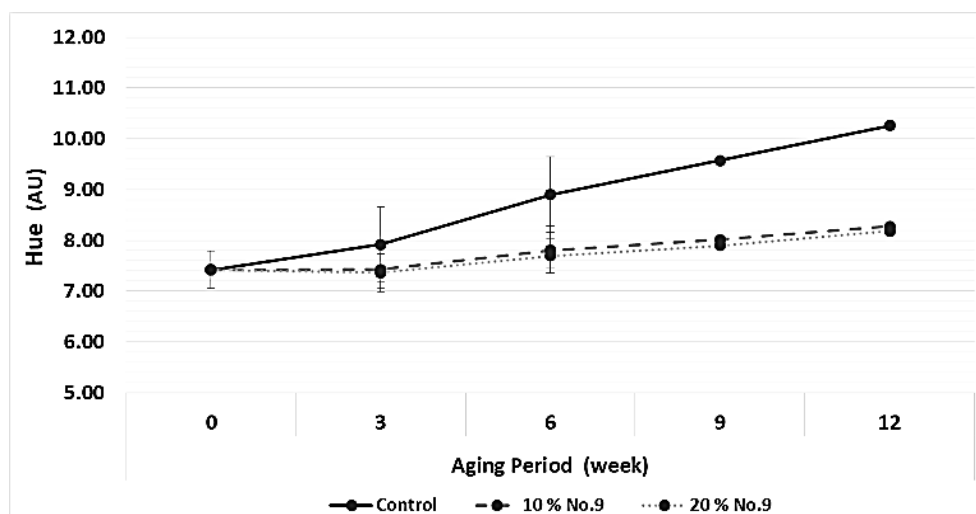
**Figure 4:** Anthocyanin content during 3 months aging of Roselle wine



**Figure 5:** Total polymeric pigments during 3 months aging of Roselle wine



**Figure 6:** Color enhancement (red pigment) during 3 months aging of Roselle wine



**Figure 7:** Color hue during 3 months aging of Roselle wine

### 3.4 Roselle wine characteristic after aging

Table 1 shows the comparison of physicochemical characteristic of Roselle wine between fresh wine and wine aged with and without *Saccharomyces sp.* No.9 after 3 months aging. The chemical analysis showed that all physicochemical components of fresh Roselle wine had changed after aged in the standard wine bottle at 10°C for 3 months.

The pH was increased while TTA and VA were decreased after 3 months aging in all treatments. Moreover lower acidity could help the wine to develop more flavor, which is the one of most appealing of good wine [4]. The alcohol contents were increased in all aged wines. The alcohol contents were increased in corresponding to the decreased in total soluble solid and reducing sugar in the aged wine. The soluble solids in wine may be broken down by chemical reactions or used by the microorganisms that remained in the wine, such as yeast. However, the Roselle wine aged with *Saccharomyces sp.* No.9 had higher percentage of alcohol than control wine (Roselle wine aged without *Saccharomyces sp.* No.9) because this aging yeast might break down some total soluble solid and use it to produce the alcohol. Moreover, the *Saccharomyces sp.* No.9 could reduce the reduction rate of anthocyanins during 3 months aging. As the results showed in Table 1, the level of anthocyanins of control aged wines was significantly lower ( $p < 0.05$ ) than the wine aged with *Saccharomyces sp.* No.9 and fresh Roselle wine. Level of anthocyanin in the aged wine was corresponded with color enhancement, which representing more stable of red color in wine aged with *Saccharomyces sp.* No.9. The significantly higher level of hue value in control aged wine represented the reduction of red pigments as well as increasing of yellow pigment (data not showed) that corresponded to the development toward the red-brown color of old wine.

In summary, the aging processes affected all physicochemical characteristics of Roselle wine. The *Saccharomyces sp.* No.9 which was added into the wine as aging yeast affected to aged wine including reducing acidity, the reduction rate of anthocyanins during 3 months aging and increaseing alcohol content. Thus, the *Saccharomyces sp.* No.9 might be used as aging yeast in

aging process to improve the wine quality for commercial red wine because it affects to all physicochemical components especially alcohol content and wine color.

**Table 1:** Physicochemical components of Roselle wine.  
(Values are means  $\pm$  SD of triplicate determinations.)

Characteristics	Based Roselle Wine (Fresh wine)	Without yeast No.9 (Aged wine)	10% of yeast No.9 (Aged wine)	20% of yeast No.9 (Aged wine)
pH	3.37 <sup>d</sup> * $\pm$ 0.052	3.47 <sup>c</sup> * $\pm$ 0.058	3.50 <sup>a</sup> * $\pm$ 0.052	3.49 <sup>b</sup> * $\pm$ 0.052
TTA (as malic acid g/L)	9.01 <sup>a</sup> * $\pm$ 0.965	7.14 <sup>b</sup> * $\pm$ 0.686	7.33 <sup>b</sup> * $\pm$ 0.529	7.25 <sup>b</sup> * $\pm$ 0.526
VA (as acetic acid g/L)	2.64 <sup>a</sup> * $\pm$ 0.201	2.01 <sup>b</sup> * $\pm$ 0.331	2.19 <sup>a b</sup> * $\pm$ 0.284	1.97 <sup>b</sup> * $\pm$ 0.181
Total soluble solid ( $^{\circ}$ Brix)	10.80 <sup>a</sup> * $\pm$ 0.231	9.85 <sup>b</sup> * $\pm$ 0.289	7.70 <sup>c</sup> * $\pm$ 0.346	7.25 <sup>d</sup> * $\pm$ 0.289
Reducing sugar (g/L)	0.23 <sup>b</sup> * $\pm$ 0.002	0.37 <sup>a</sup> * $\pm$ 0.036	0.15 <sup>c</sup> * $\pm$ 0.026	0.13 <sup>c</sup> * $\pm$ 0.011
% Alcohol	8.93 <sup>c</sup> * $\pm$ 0.660	10.58 <sup>b</sup> * $\pm$ 0.403	12.55 <sup>a</sup> * $\pm$ 0.526	12.48 <sup>a</sup> * $\pm$ 0.479
Total phenolics (mg/L CE)	1392.89 <sup>a</sup> * $\pm$ 57.117	1082.40 <sup>b</sup> * $\pm$ 29.322	1106.73 <sup>b</sup> * $\pm$ 54.373	1076.82 <sup>b</sup> * $\pm$ 20.947
Anthocyanin content (mg/L M3G)	255.50 <sup>a</sup> * $\pm$ 9.950	62.00 <sup>c</sup> * $\pm$ 8.165	103.00 <sup>b</sup> * $\pm$ 8.485	107.75 <sup>b</sup> * $\pm$ 3.948
Color enhancement (AU)	4.75 <sup>a</sup> * $\pm$ 0.477	3.79 <sup>c</sup> * $\pm$ 0.115	4.07 <sup>b</sup> * $\pm$ 0.214	4.10 <sup>b</sup> * $\pm$ 0.257
Hue (AU)	7.42 <sup>b</sup> * $\pm$ 0.365	10.26 <sup>a</sup> * $\pm$ 0.744	8.27 <sup>b</sup> * $\pm$ 0.476	8.18 <sup>b</sup> * $\pm$ 0.337

\* Values with the “ \* ” attached are significantly different between sample ( $p > 0.05$ ).

\*\* Values with the same letters attached are not significantly different ( $p > 0.05$ ).

## 4 CONCLUSIONS

Through the statistical analysis, it was found that *Saccharomyces sp.* No.9 addition for aging could affect on the physicochemical characteristics of aged Roselle wine after 3 months of aging compared to control wine. The aged wine with *Saccharomyces sp.* No.9 was significantly higher in alcohol content, anthocyanin contents, and color enhancement. Furthermore, the aged wine was also significantly lower in reducing sugar and hue. However, the different percentages of yeasts added had no effect on the physicochemical components of aged Roselle wine after 3 months of aging

The result provided information that *Saccharomyces sp.* No.9 could sustain level of anthocyanin in the wine after 3 months of aging. The higher anthocyanin in the wine aged with *Saccharomyces sp.* No.9 could be beneficial to the color of the wine as showed in the analysis of hue and color enhancement. Therefore, the application of *Saccharomyces sp.* No.9 for wine aging could possibly improve characteristics of Roselle wine which will be beneficial to the industry.

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## **EXTRACTION AND EVALUATION OF LIMONOIDS FROM CITRUS SEEDS AND THEIR ANTIOXIDANT CAPACITY**

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### **ABSTRACT**

Citrus seeds contain high amounts of limonoids which have high health benefits. In this study, total limonoid aglycones and antioxidant capacities of extracts from seeds of Thanh Tra pomelo were investigated. The limonoids were extracted using different organic solvents including ethyl acetate, acetone, methanol and dichloromethane and total limonoid content was determined by colorimetric methods using DMAB indicator reagent. The antioxidant capacities of the extracts were determined by both DPPH and ABTS radical scavenging assays. The results showed that total limonoid concentration of the extract was the highest using ethyl acetate solvent ( $44.04 \pm 2.79$  mg/ml), followed by methanol ( $41.73 \pm 1.55$  mg/ml), acetone ( $39.92 \pm 2.48$  mg/ml) and dichloromethane ( $19.58 \pm 1.61$  g/ml). However, antioxidant capacity of the pomelo seed extracted by methanol was the highest, followed by acetone, ethyl acetate and dichloromethane. The pomelo seed extract using methanol possessed 38.5% DPPH scavenging, 50.7% ABTS scavenging and equivalently 10.6 mM ascorbic acid. As the results, pomelo seed was considered to be a good source of bioactive compounds and antioxidants.

**Keywords:** limonoids; extraction; antioxidant; glucoside; aglycone

### **1 INTRODUCTION**

Citrus fruits have been recognized as one of the most healthful source in human diet. Recent studies on bioactive compounds, specifically limonoids, have proved their major role in preventing chronic diseases. Citrus limonoids occur in high concentrations as aglycones and glucosides in fruit tissues and seeds [1]. Significant progress has demonstrated the number of biological activities of these compounds including anti-inflammatory, antiviral, antiproliferative

and anticarcinogenic activities *in vivo* system. Limonoids are reported to be nontoxic to human, animals and noncancerous cells of mammals [2].

Several natural antioxidants have already been identified in different kinds of plant materials including citrus seeds [3]. Among 56 limonoids have been identified in citrus, limonin is the first characterized compound of these phytochemicals knowing as a constituent of citrus since 1841. The studies *in vivo* and *in vitro* indicated that limonin is a potential bioactive compound and it exhibits a number of significant biological activities. Limonin has been involved in the inhibition of development of oral tumors in hamster cheek pouch model and has been found to influence GST enzyme activity in liver and small intestine mucosa of rodent models [4]. Treatment with limonin has showed a considerable effect in inhibition of DMBA-induced neoplasia in experimental mice. Besides anticancer activity limonin has antimicrobial activities [5], cholesterol lowering [6], antiviral activities and acts as protective agent against low-density lipoprotein (LDL) oxidation.

The purpose of this project is to investigate effects of different solvent extraction on total limonoid and limonin contents and their antioxidant capacity of extracts from Thanh Tra pomelo's seeds.

## **2 MATERIALS AND METHODS**

### **2.1 Material**

Thanh Tra pomelo's seeds were collected from Tien Giang Province and dried to achieve moisture content below 10%. Then seeds were ground into powder and stored in desiccator for further analysis.

### **2.2 Analytical methods**

#### *2.1.1 Sample preparations*

Portion of seed sample was firstly extracted with n-hexane by a Soxhlet extractor to remove oily materials in 8 hours.

#### *2.1.2 Limonoid extraction*

The procedure was carried out based on Soong & Barlow's method [7]. One gram of seed sample was refluxed with 20 ml of each solvent (ethyl acetate, acetone, methanol and dichloromethane) at 70°C for 20 minutes. The mixture was then centrifuged at 8,000 rpm at 4°C xfor 15 minutes. The extraction was repeated for three times and supernatants were filtered and evaporated by rotary evaporator at 40°C. Then the residue was made up with 10 ml of acetonitrile and kept for further measurement.

### 2.1.3 Measurement of total limonoid equivalents using DMAB reagent

The colorimetric methods using DMAB indicator reagent for quantification of total limonoid was employed by method of Andrew & Phil. [8]

Preparation of DMAB indicator reagent: 24 ml of Perchloric acid 70% was combined with 30ml of acetic acid (glacial) to prepare stock acid solution. The DMAB reagent was freshly prepared by dissolving 0.56 g of DMAB in 15 ml of stock acid solution.

Measurement of total limonoids: 330  $\mu$ l of DMAB indicator and stock acid solution was added into 220  $\mu$ l of sample and then incubate at room temperature for 30 minutes. Next, the absorbance was measured at 503 nm by UV-Vis. Limonin standard was prepared in acetonitrile (0.1 mg/ml). The results were expressed as  $\mu$ g/g.

### 2.1.4 HPLC analysis of limonin

An aqueous (20  $\mu$ l) of reconstituted sample in acetonitrile was injected to C18 reversed phase column and eluted isocratically at 1ml/min flow rate using a mobile phase composed of 3 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient elution was conducted, starting at 85% of solvent A, reduction to 77% in 5 min, 74% after 25 min, further reduction to 60% at 30 min and completing the gradient at 54% at the end of 45 min. The column was equilibrated for 5 min with 85% solvent A and 15% solvent B before next run [9]. The elution was monitored at UV wavelength 210 nm and carried out at room temperature.

### 2.1.5 Antioxidant capacity by DPPH radical scavenging assay

The DPPH radical scavenging activity was measured by Liyana-Pathirana & Shahidi's [10] method. Briefly, 3.9 ml of 0.075 mM DPPH solution was mixed with 0.1 ml of sample. The mixture was vortexed and kept in dark at room temperature. The absorbance was recorded at 515 nm after exactly 30 minutes. Blank solution was prepared from 0.1 ml of methanol and the absorbance was read at  $t = 0$ . The scavenging of DPPH was calculated according to the following equation:

$$\% \text{DPPH scavenging} = \left\{ (\text{Abs}_{t=0} - \text{Abs}_{t=30}) / \text{Abs}_{t=0} \right\} \times 100 \quad (1)$$

Where:  $\text{Abs}_{t=0}$  = absorbance of DPPH radical + methanol at  $t = 0$  min

$\text{Abs}_{t=30}$  = absorbance of DPPH radical + methanol at  $t = 30$  min

### 2.1.6 Antioxidant capacity by ABTS cation radical-scavenging assay

The ABTS radical-scavenging activity was followed by Robert et al. [11]. ABTS cation chromophore was generated by reacting 7 mM ABTS with 2.45 mM potassium and allowing the mixture to stand in the dark for at least 16h at room temperature. The mixture was then diluted with absolute ethanol and measured at 734 nm to give an absorbance of  $0.70 \pm 0.02$ . After

addition of 1 ml of ABTS ethanolic solution to 10 µl of sample, the absorbance was taken exactly 1 minute after initial mixing and up to 6 minutes. Ascorbic acid was used as standard. The percentage inhibition of absorbance was plotted as a function of concentration of antioxidant and was calculated by the following formula:

$$\% \text{ABTS scavenging} : \left( 1 - \frac{\text{Abs}_f}{\text{Abs}_0} \right) \times 100 \quad (2)$$

Where:  $A_0$  = absorbance of solution without the presence of sample/standard

$A_f$  = absorbance of solution after addition of sample/standard

### 3 RESULTS AND DISCUSSION

#### 3.1 Limonin equivalents of total limonoid extracts

Total limonoid aglycone and limonin content of pomelo seeds were indicated in Table 1. Pomelo witnessed a remarkable limonoids concentration in ethyl acetate fractions ( $44.04 \text{ mg/g} \pm 2.79$ ) which was followed by methanol ( $41.73 \text{ mg/g} \pm 1.55$ ) and acetone ( $39.92 \text{ mg/g} \pm 2.48$ ). Unlike other solvents, dichloromethane was the least effective one applied in limonoids extraction. Although pomelo sample demonstrated the highest total limonoid content in ethyl acetate fraction, limonin content extracted by methanol reached the highest concentration ( $13.72 \text{ mg/g}$ ), followed by limonin in the extracts with ethyl acetate and acetone. The limonin content in the extract with dichloromethane was the lowest compared to other solvents.

**Table 1:** Total limonoid aglycone and limonin content of citrus seed (mg/g)

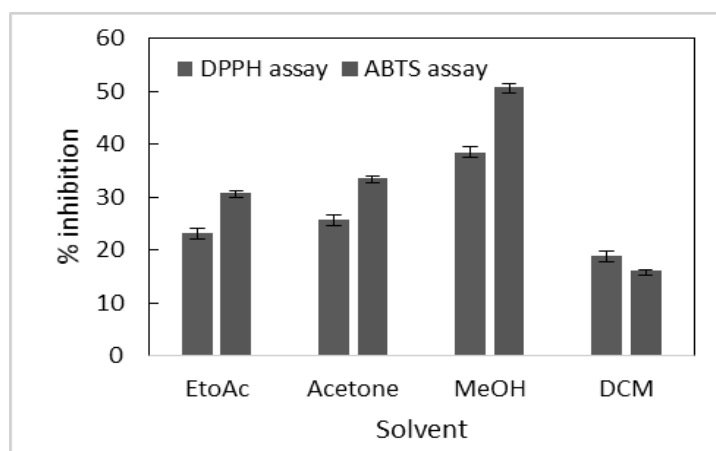
Solvent	Total limonoid aglycone (mg/g)	limonin content (mg/g)
EtoAc	$44.04 \pm 2.79^b$	$8.32^b$
Acetone	$39.92 \pm 2.48^b$	$8.03^b$
MeOH	$41.73 \pm 1.55^b$	$13.72^c$
DCM	$19.58 \pm 1.61^a$	$4.16^a$

*Values (means of triplicate measurements) with different letters within a column are significantly different ( $p < 0.05$ ).*

#### 3.2 DPPH and ABTS radical scavenging assay

Figure 1 expressed the radical scavenging ability of pomelo seeds varied with the solvent applied. Generally, antioxidant capacity detected by ABTS assay was significantly higher comparing to DPPH assay. In addition, the antioxidant activity determined by ABTS assay followed the same trend with the results obtained from DPPH assay when comparing with extracts from different solvents. While seed extracts in methanol (DPPH assay  $38.53 \pm 0.45\%$ ; ABTS assay  $50.77 \pm$

0.6%) demonstrated the highest antioxidant capacity comparing to other solvents, fractions extracted with dichloromethane performed the lowest activities (DPPH assay  $18.84 \pm 0.32$ ; ABTS assay  $16.13 \pm 0.21$ ). The percentage of inhibition of extracts using ethyl acetate and acetone is similar in both methods. The different in another factor which affects the result is the nature of the solvent. Some solvents can yield higher amount of limonoids, therefore, these fractions perform better antioxidant capacity. Acetone is the best solvent for limonoid aglycones extraction [13], whereas methanol is preferred for highly polar glucosidal limonoids [14]. From the obtained results, dichloromethane appears to be the least effective among other solvents for solvent extraction technique due to their low yield of limonoids.



**Figure 1:** Antioxidant capacity (% inhibition) of extracts using DPPH and ABTS assay

Table 2 showed the concentration of pomelo's seed samples expressed in (nM) ascorbic acid equivalent corresponding (AEAC). The extent of inhibition of absorbance of ABTS cation is considered as function of concentration antioxidant to measure AEAC as function of time [15]. Thus the determinants of the antioxidant activity are the extent of reduction and rate of reduction of the radical. The obtained results show that extracts using methanol had the highest AEAC value ( $10.06 \pm 0.13$  nM at 6 min); that for the highest concentration in antioxidant compounds.

Methanol has polarity index is 5.1 [16], it is used for extraction most of the polar compounds [17; 18; 19]. Therefore, methanol is commonly used for extraction of bioactive compounds. Although methanol is highly toxicity [20], it can easy to be evaporated because of low-boiling point [21] which is  $64.7^{\circ}\text{C}$  at 760 mm Hg [22]. Moreover, this solvent in the extract was also removed under reduced pressure at  $40^{\circ}\text{C}$  by using rotary evaporator [23]. Therefore, methanol will be clearly removed after evaporation with suitable condition and not cause toxicity to the products.

**Table 2:** Antioxidant activity as ascorbic acid equivalents (mM) at specific time

Solvent	1 min	6 min
EtoAc	5.77±0.10 <sup>b</sup>	5.91±0.08 <sup>b</sup>
Acetone	6.31±0.12 <sup>c</sup>	6.47±0.07 <sup>c</sup>
MeOH	9.50±0.12 <sup>d</sup>	10.06±0.13 <sup>d</sup>
DCM	2.78±0.04 <sup>a</sup>	2.82±0.04 <sup>aA</sup>

Values (means of triplicate measurements) with different letters within a column are significantly different ( $p < 0.05$ ). \* 100 times of dilution.

## 4 CONCLUSIONS

In this study, using methanol to extract limonoids demonstrated higher antioxidant capacity in pomelo seed. This finding could be applied in producing food additives and disease management products to fulfill a risen demand for citrus limonoids due to their significant bioactivity in structure-activity relationship and health promoting properties.

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## **INSTANT LACTIC ACID BACTERIA CULTURE FOR MAKING VEGETABLE PROBIOTIC BEVERAGES FROM BITTER GOURD (*MOMORDICA CHARANTIA L.*) JUICE**

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### **ABSTRACT**

Fruit based probiotic beverages can be advantageous for our health and good for metabolism. Bitter gourd (*Momordica charantia L.*) is a kind of fruit that contains a lot of vitamins, minerals, antioxidants, and bioactive compounds which can support our health. Bitter gourd juice fermentation using lactic acid bacteria can enhance the nutrition compound, shelf-life, and some of sensory properties of the juice. Lactic acid bacteria used in this research were *Lactobacillus fermentum* LLB3 and *Lactobacillus pentosus* LLA18 from the isolated strains of the second year PUPT (Penelitian Unggulan Perguruan Tinggi) research. Variety of bitter gourd used in this research was “Kambas”. The aim of this research was to examine the effect of using lactic acid bacteria toward the physicochemical and microbiological characteristics of fermented “Kambas” bitter gourd juice as the probiotic beverages. In this fermentation method, 2 different durations of fermentation were examined, they were 24 and 48 hours. The viability of lactic acid bacteria was determined by using total plate count (TPC) method and expressed as CFU/ml. DPPH radical-scavenging activity, pH value, sucrose concentration were measured at different time of fermentation (24 and 48 hours). All of the data were analyzed by Microsoft Excel 2010. The results showed that viable counts of probiotics increased during 24 hours of fermentation and decreased after 48 hours of fermentation. The pH and total dissolved solid or sucrose concentration of probiotic beverages decreased gradually during fermentation.

**Keywords:** Bitter gourd, lactic acid bacteria, antioxidant activity, probiotic beverages

### **1 INTRODUCTION**

Nowadays, people pay a lot of attention to the relation between food and health. People currently expect something more than delicious food, therefore foods with health promoting properties, or as we called functional foods, have shown a remarkable growth over the last few years [33]. The growing interest in functional foods with health benefits has led to the development of new

functional beverages, which can provide a health promotion and disease prevention by reducing the increasing burden on health care system through a continuous preventive mechanism [8].

Beverages are considered as a good medium for the supplementation of nutraceutical compounds, such as herbal extracts [8]. Probiotic beverages have been fermented by probiotic bacteria like *Lactobacillus* or *Bifidobacterium*. Probiotics bacteria may produce many compounds that can inhibit the pathogen's growth such as lactic acid and acetic acid. These compounds can lower the pH and inhibit the growth of pathogens, but they also can be toxic to some microbes. Because of that, probiotics have many benefits for human health such as preventing lactose intolerance and milk allergy for person from diarrhea, preventing constipation, lowering blood cholesterol, boosting immune, and preventing cancer [24].

Probiotic beverages are available on the market today and usually in the form of fermented milk and yoghurt. However, there is an increasing of vegetarianism consumer throughout developed countries, therefore a demand for vegetarian probiotic product is also increasing. In current years, a demand for non-dairy-based probiotic products has increased due to many problems, such as lactose intolerance and cholesterol content associated with the consumption of fermented dairy products [27]. These problems lead to the use of vegetables as an alternative for the production of probiotic foods due to their nutritive values and large distribution. Foods such as fruits, grains, and vegetables are reported to contain a large variety of antioxidant components, including phytochemical, such as phenolic compounds. They are considered as beneficial compounds for human health and can decrease the risk of degenerative diseases by inhibiting the macromolecular oxidation and reduction of oxidative stress [27].

*Momordica Charantia L.* (Bitter Gourd) has long been used as a food and medicine. From the research about antioxidant properties of bitter gourd which has been evaluated by Hamissou [9] in the journal with the title "Antioxidative Properties of Bitter Gourd (*Momordica charantia*) and Zucchini (*Cucurbita pepo*)", bitter gourd contains high concentration of antioxidants. Comparing with other vegetables like carrot, tomato, celery, cabbage, green pepper, onion, and winter melon, bitter gourd has richer antioxidants component [28]. Moreover, bitter gourd also has antidiabetic compound that is known as charatin compound. From Altinterim's research [3] with the title "Bitter Melon (*Momordica charantia L.*) and the Effects of Diabetes Disease", bitter gourd has the ability to reduce blood glucose and enhance insulin sensitivity, therefore, it can be used as antidiabetic, especially for diabetes mellitus type 2. Unfortunately, the consumption of this fruit is still rare. It is because this fruit has a very bitter taste. Sometimes, if the cooking method is not appropriate, the bitter taste still remains in the food. People nowadays like to consume healthy processed food to maintain their health. Therefore, bitter gourd can be consumed as probiotic beverages by fermenting it.

Fermentation is the method for making probiotic beverages. Fermentation can enhance the nutrient values of food through the biosynthesis of vitamins and amino acids by increasing the

protein and fibre digestibility. For fermenting food products, we need some microorganisms that can do the fermentation process. Mostly, the microorganisms that are used for fermenting food are yeast and lactic acid bacteria (LAB) [15]. Lactic acid fermentation can help to improve nutrition, safety, shelf-life and also sensory properties of vegetables. Therefore it also can reduce the use of food additives regarded to its ability to preserve food products from spoilage and improve the organoleptic properties of foods. Several *Lactobacillus* species have been proven to develop a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens, and reduction of blood cholesterol [27].

The research about the effect of lactic fermentation on antioxidant activity in herbal tea by Ibrahim [12] explained that fermentation is the oldest and economical method to preserve food or enhance the nutrition values. Fermentation of herbs by lactic acid bacteria can increase the antioxidant activity. The results from the research of Ibrahim et. al (2014) [12] showed that fermented herbal tea has a higher antioxidant activity than the fresh one. The antioxidant activity increased from 45% to 85%. Therefore, the fermentation of bitter gourd juice can produce probiotic beverage that has more functional properties with higher concentration of antioxidant than the unfermented bitter gourd juice and also has antidiabetic compound that is known as charantin.

The research about fermentation of “kambas” bitter gourd (*Momordica charantia* L.) juice using instant lactic acid bacteria for making probiotic beverages still had some limitations. Therefore, the objectives of this study were to investigate the suitability of “kambas” Bitter Gourd as a raw material, and the effects of lactic acid fermentation on antioxidant activity, pH, sugar concentration, the viability of LAB, bacteriocin activity, and antimicrobial activity of fermented bitter gourd juices.

## 2 MATERIALS AND METHODS

### 2.1 Materials

The materials in this research were fresh “Kambas” bitter gourds (*Momordica Charantia* L.) purchased from local market, instant lactic acid bacteria (culture de yogurt yogourmet) consist of skimmed milk powder, sucrose, and active bacterial culture of *Lactobacillus bulgaricus*, *Streptococcus Thermophilus*, and *Lactobacillus acidophilus*, *Eschericia coli* (fnc 0091), *Staphylococcus aureus* (fnc 47), *Salmonella typhimurium* (fnc 0187), MRS-broth (MRSB), MRS-agar (MRSA), CaCO<sub>3</sub>, nutrient agar (NA), nutrient broth (NB), tomato extract, sterile aquadest, 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH), methanol 98%, hexane, ethanol, aquadest, alcohol, 1M HCl, and 1M NaOH.

### 2.2 “Kambas” Bitter Gourd Juice Preparation

Fresh “Kambas” bitter gourd was purchased from local market, Jatingaleh. All fruits were washed, chopped into small pieces and blended using a juicer eliminating seeds. The fresh juice

was filtered using a filter fabric to collect the filtrates and remove the solid particles. The filtrates were kept in a sterile bottle and then were pasteurized at 70°C for 5 minutes.

### **2.3 Inoculum of Lactic Acid Bacteria Preparation**

One milliliter of stock culture of instant lactic acid bacteria was put into 9 milliliters of MRSB media + 1 milliliter of tomato extract. This culture was incubated for 48 hours at 37°C. After that, the culture was centrifuged at 3000 rpm for 15 minutes. The supernatant were removed from the tube and 9 ml of MRSB media were put into the centrifuge tube (renew the media).

This culture was incubated for 24 hours at 37°C. In the next day, the culture was centrifuged again at 3000 rpm for 15 minutes and the supernatant was removed. After that, 6 ml of MRSB media were put into the centrifuge tube and the inoculum was ready to be used. Two ml of instant lactic acid bacteria inoculum culture was prepared. The culture was inoculated into 18 ml of bitter gourd juice samples. The inoculum was incubated in incubator at 37°C for 24 hours.

### **2.4 Fermentation of “Kambas” Bitter Gourd Using Lactic Acid Bacteria**

Fermentation experiments were conducted in 250 ml Schott Duran Bottles, containing 180 ml of pasteurised Kambas Bitter Gourd juice. All the bitter gourd juice samples were added with 20 ml inoculum of lactic acid bacteria. Then, the bitter gourd was fermented in the incubator at 37°C for 48 hours. Samples were taken at 24 hours intervals and the viability of lactic acid bacteria in “Kambas” Bitter Gourd Juice was determined by total plate count and expressed as colony forming units (CFU/ml). DPPH radical-scavenging activity, pH measurement, and determination of sugar concentration were measured at the different time of fermentation (24 and 48 hours).

### **2.5 Total Plate Count**

#### *2.5.1 Making Sterilized Media*

MRSA media contain 1% of CaCO<sub>3</sub> was made by dissolving 68,17 gram of MRSA powder and 10 gram of CaCO<sub>3</sub> in 1000 ml of sterile aquadest [21]. The solution was heated in a hotplate at 250°C and was stirred using a magnetic stirrer until the solution were completely boiled. The media was sterilized at 121°C for 60 minutes.

#### *2.5.2 Spread Plate Methods*

One milliliter of the inoculum suspension were pipetted by an Acura Fix Micropipette Socorex into a dilution tube containing 9 ml of sterile aquadest. This tube was vortexed for about 5 seconds. After vortexing, 1 ml of this volume was removed and placed into a second dilution tube containing 9 ml of sterile aquadest [10]. This process was repeated exactly until the dilution reached 10<sup>-6</sup>. Agar plate was made by pouring 11 ml of sterilized media solution into a sterile plates and let to solidify. The dilution tube was vortexed for approximately 5 seconds and 0,1ml aliquots of the sample were picked up using a pipette and plated into three triplicates. To avoid

the loss of water from agar, the plates were wrapped in plastic bags during the incubation [22]. The plates were incubated at 37°C for 48 hours. After that, colonies were counted by Stuart Digital Colony Counter and colony with clear zones were identified as lactic acid bacteria. For computation, total colony per plate was divided by dilution factor ( $10^{-6}$ ) and it was expressed as CFU/ml.

$$\frac{\text{CFU}}{\text{ml}} = \text{Total Colony} \times \frac{1}{\text{dillution factor}} \times 10$$

### 2.5.3 Determination of Free Radical Scavenging Activity by 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH)

The free radical scavenging activity was determined by the method of Suja *et al.* [29] with some modifications. Firstly, Methanolic DPPH solution with the absorbance of  $1.00 \pm 0.01$  unit at 515 nm wavelength was prepared freshly before use. Each fresh sample (0,1 ml) with different type of lactic acid bacteria and different type of media was added with 3,9 mL of DPPH solution in methanol. Absorbance at 515 nm was taken after allowing the solution to stand for 30 minutes. All of the samples and blank solution (3,9 ml DPPH + 0,1 ml methanol) were monitored at 515 nm wavelength by spectrophotometer. Each sample was carried out five times. The percentage of DPPH scavenging activity was determined as follow:

$$\text{DPPH Scavenging Activity} = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{\text{absorbance of blank}} \times 100\%$$

## 2.6 Bacteriocin and Antimicrobial Inhibitory Activity Test

One milliliter of each sample that had been fermented for 24 and 48 hours was placed into Eppendorf. The solution and cells were separated by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was collected in sterile tube and pellet was discarded. The cell-free supernatant was adjusted to pH 6.0 by 1M NaOH and 1M HCl. The purpose of adjusting the cell-free supernatant to pH 6.0 was to prevent the inhibitory effect of lactic acid [31]. Bacteriocin activity was analyzed by agar well diffusion method. From this method, clear zone around the well will be obtained. A 10 µl of pathogen inoculum (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella thypimurium*) equivalent to  $10^6$  CFU/ml (Mc Farland 3) were inoculated with 10 ml of NA medium using pour plate method and let to solidify. After the media become hard, the plate was divided into 5 sections (1 section for negative control and 2 section for neutralized samples with 2 times replication and 2 section for samples with 2 times replication). Twenty µl of sample (with or without neutralization) were inoculated in 7 mm diameter wells that had been cut in each section. Sterilized aquadest was used as a negative control. Diameter of the clear zone formed around the well were measured by vernier calipers after incubation at 37°C for 24 hours. Inhibitory activity of bacteriocin was expressed as AU (Arbitrary Unit) per ml, defined as the unit



area of inhibition zone per unit volume of sample tested bacteriocins (mm<sup>2</sup>/ml) [30]. Bacteriocin activity can be calculated using the following formula (Usmiati & Marwati, 2009):

$$\text{Bacteriocin Activity} \left( \frac{\text{mm}^2}{\text{ml}} \right) = \frac{Lz - Ls}{V}$$

Description:

Lz: clear zone area (mm<sup>2</sup>)

Ls: well area (mm<sup>2</sup>)

V: sample volume (ml)

## 2.7 pH Measurement

pH of the fermented “Kambas” bitter gourd were measured by digital pH meter (EC-pHTestr30-KIT). pH measurement was done three times (triplicate) for each sample. [7]

## 2.8 Determination of Total Dissolved Solid (Expressed as Sucrose)

Total dissolved solid expressed as sucrose concentration of the samples were determined by brix refractometer (Hanna Instruments Sucrose Refractometer) [17]. The sucrose concentration was expressed in degrees of Brix (40° Brix is equivalent to a sugar content of 40%). The term of apparent brix was used because bitter gourd juice is a non-sucrose based product [5].

# 3 RESULTS AND DISCUSSION

## 3.1 Viability of Lactic Acid Bacteria (LAB) in Fermented Bitter Gourd Juice

**Table 1:** Viable Lactic Acid Bacteria During Fermentation

Time of Fermentation (hour)	Viable Counts (CFU ml <sup>-1</sup> )
0 (Fresh Bitter Gourd)	0.23 ± 0.03 x 10 <sup>6</sup>
24	4.33 ± 0.75 x 10 <sup>6</sup>
48	0.07 ± 0.008 x 10 <sup>6</sup>

Viable count (CFU mL<sup>-1</sup>) of fresh bitter gourd and instant lactic acid bacteria strain in bitter gourd juice drink during fermentation at 37°C for 0, 24, and 48 hours are presented in Table 1. For probiotics to be effective, scientists suggested that there is a minimum of 10<sup>6</sup>–10<sup>7</sup> CFU of probiotic bacteria per milliliter of product at the time of consumption which has been achieved by two strains [14]. Based on existing standards and from a health view-point, it is very important that probiotic strains can retain their viability and functional activity throughout the fermentation of the product. Some probiotic strains do not grow well in milk. In such cases the presence of plant-based ingredients may improve the growth of probiotic cultures in milk such as tomato



juice, peanut milk, soy milk, bitter gourd, carrot and cabbage juice [20]. In Table 1, the viable count of LAB in fresh bitter gourd without fermentation was  $0.23 \times 10^6$  CFU mL<sup>-1</sup>, whereas the viable cell count of instant lactic acid bacteria in the fermented bitter gourd juice was  $4.33 \pm 0.75 \times 10^6$  CFU mL<sup>-1</sup> after 24 hours of fermentation and  $0.07 \pm 0.008 \times 10^6$  CFU mL<sup>-1</sup> after 48 hours of fermentation. The changes of viable cell counts showed that the number of cell after 24 hours of fermentation increased, and after 48 hours of fermentation, the number of cell decreased. Maintaining the viability and the activity of probiotics in foods to the end of fermentation time are two important criterias to be fulfilled in juices, where low pH represents a drawback. Several strains of genus *Lactobacillus* can grow in fruit matrices due to their tolerance to acidic environments [23].

The time courses of lactic acid fermentation of bitter gourd juice by instant lactic acid bacteria is presented in Table 1. Based on the data, instant lactic acid bacteria grew rapidly on vegetable juice and reached the highest number of probiotic bacteria (CFU mL<sup>-1</sup>) nearly  $4.33 \pm 0.75 \times 10^6$  CFU mL<sup>-1</sup> probiotic bacteria after being fermented for 24 hours at 37°C. Beyond 24 hours, fermentation did not result in a significant increase in viable cell count of probiotic bacteria. It is probable that instant lactic acid bacteria require some essential growth nutrients that are deficient in the vegetable juice [6].

Although juices contain some essential nutrients (minerals, vitamins, dietary fibers, antioxidants), there are some strong factors that could limit probiotic survival in juices which are food parameters (pH, titratable acidity, molecular oxygen, water activity, presence of salt, sugar and chemicals, hydrogen peroxide, bacteriocins, artificial flavoring and coloring agents), processing parameters (heat treatment, incubation temperature, cooling rate, packaging materials and storage methods, oxygen levels, volume), and microbiological parameters (strains of probiotics, rate and proportion of inoculation). pH is one of the most important factors affecting the survival of probiotics. *Lactobacilli* are generally acidic-pH resistant and can survive in juices with pH ranging from 3.7 to 4.3 [12].

### 3.2 Antioxidant Activity of Fermented Bitter Gourd Juice

**Table 2:** Antioxidant Activity of Fermented Bitter Gourd Juice

Fermentation (hour/s)	Antioxidant Activity (%)
Fresh Bitter Gourd (0)	$85.82 \pm 0.01$
Pasteurised	$88.62 \pm 0.01$
24	$85.49 \pm 0.02$
48	$77.27 \pm 0.03$

The results of antioxidant activity of bitter gourd juice fermented with instant lactic acid bacteria at different fermentation time are shown in Table 2. Fermentation technology can prolong the

shelf-life and enhance nutritional and organoleptic qualities of food. During fermentation, many biochemical changes occurred, leading to an altered ratio of nutritive and anti-nutritive compounds. Therefore, product properties such as bioactivity were affected significantly [11]. Bitter melon is a good source of phenolic compounds, such as gentisic acid (2, 5-dihydroxyl benzoic acid), gallic acid, catechins, epicatechin and chlorogenic acid [8]. As reported by Hur *et al.* [11], during the fermentation there was the bioconversion of the conjugated forms of phenolic compounds into their free forms, so their antioxidant activity was enhanced.

The results of antioxidant activity determined as free radical scavenging activity decreased after 24 hours of fermentation on bitter melon juice fermented by *Lactobacillus fermentum* strain and instant LAB, while antioxidant activity of bitter melon juice fermented with *Lactobacillus pentosus* strain increased after being fermented for 24 hours. After 48 hours of fermentation, antioxidant activity decreased on bitter melon juice fermented with *Lactobacillus pentosus* strain, although it was slightly higher than that of the bitter melon juice fermented with *Lactobacillus fermentum*. Moreover, the antioxidant activity of bitter melon juice fermented with instant LAB kept decreasing through the fermentation process. The range of antioxidant activity was between  $86.10\% \pm 0.05$  and  $89.39\% \pm 0.01$  at the beginning of fermentation,  $80.59\% \pm 0.11$  and  $89.03\% \pm 0.02$  after 24 hours of fermentation,  $77.27\% \pm 0.03$  and  $82.97\% \pm 0.01$  after 48 hours. This result showed that different Lactic Acid Bacteria strain can make change in optimum fermentation time. Hur *et al.* (2014) [11] also indicated that the antioxidative activity could be affected by microbial strains present in the fermentation of plant-based foods. Therefore, the effects of fermentation on the antioxidant activity depends on the species of microorganism. Besides Lactic Acid Bacteria strain, antioxidant activity of Fermented Bitter Melon Juice also were effected by lactic acid produced, mode of glucose fermentation, high salt concentrations, growth ability at different temperature, and acid or alkaline tolerance [33].

Antioxidant activity is the total capacity of eliminating free radicals both in the cell and in food. Refers to Katina *et al.* (2007) and Hur *et al.* (2014) [11] fermentation improved antioxidative activity by increasing flavonoids released from plant-based foods. The fermentation itself induces the destruction of plant cell walls, which liberates or induces the synthesis of various bioactive compounds. The presence of lactic acid bacteria in controlled fermentation process leads to conversion simple phenolic and the depolymerization of high molecular weight phenolic compounds [11]. Total phenolic compound increased after fermentation, therefore an increasing of antioxidative activity was observed. Besides the increase of antioxidant activity during fermentation time, there was also the decrease of antioxidant activity, which were probably caused by the degradation of phenolic compounds, thus phenolic loss was responsible for the decrease of antioxidant activity. This result showed that some fermentation processes had negative effect on the antioxidant activities.

### **3.3 Bacteriocins and Antimicrobial Inhibitory Activity in Fermented Bitter Gourd Juice**

#### *3.3.1 Bacteriocins Inhibitory Activity of Fermented Bitter Gourd Juice*

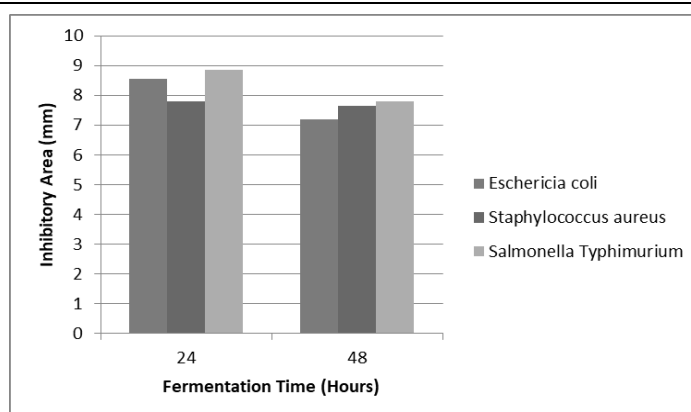
The result of analysing bacteriocins inhibitory activity showed that all of the fermented bitter gourd juice with different fermentation times (24 hours and 48 hours) didn't produce bacteriocins, whereas the fresh bitter gourd juice did and was able to inhibit the growth of pathogen bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. This result had been evaluated in previous research. Bitter gourd had antibacterial and antifungal properties to *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The result had been done by four different methods such as stroke disc diffusion, pour plate method, well diffusion, and streak plate methods [13]. According to well diffusion method from those research, bitter gourd extract had an ability to produce inhibition zone around the well with the inhibition area about 5-20 mm<sup>2</sup>. But in this research, bacteriocin inhibitory activity of fresh bitter gourd juice produced inhibition area about 118,76 – 147,34 mm<sup>2</sup>. In fermented samples, bacteriocins were not produced because the production of bacteriocins depended on several factors such as medium composition, surfactant, temperature, and pH [1].

As stated in the journal by Abbasiliasi (2017) [1], the production of bacteriocins was not only influenced by the type of carbon and nitrogen of the media but also by the ratio and concentration of them. In this case, mostly the nutritions of the bitter gourd juice were used for the growth of the lactic acid bacteria and only a few of them were used for producing bacteriocins. Besides the composition of media, the addition of surfactant also could improve the production of bacteriocin because it could enhance the sensitivity of the indicator strain and the using proteinaceous compound to stabilize the production of bacteriocin. However, in this research, surfactant was not added. Temperature and pH also become the main factors of bacteriocin production. The optimal temperature or pH for bacteriocin production are not the same with the optimal temperature and pH for bacterial growth. From another research, the slow growth rate of amylovorin L471 at low temperature contributed in the release of more energy for producing bacteriocins [1]. The growth rate was low and increased the availability of some metabolites to produce bacteriocins. Commonly, bacteriocins can be produced optimally at pH 5.5-6 with temperature slightly lower than the optimal growth temperature. With some bacteria, the production of bacteriocins can be increased in low pH [1].

### 3.3.2 Antimicrobial Activity of Fermented Bitter Gourd Juice

**Table 3:** Diameter (mm) of Antimicrobial Inhibitory Activity to *Eschericia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* after 24 and 48 Hours of Fermentation

Fermentation Time (hour/s)	Antimicrobial Activity (mm)		
	<i>Eschericia coli</i>	<i>Staphylococcus aureus</i>	<i>Salmonella Typhimurium</i>
Fresh Bitter Gourd (0)	13,50 ± 0,28	12,60 ± 0,14	14,30 ± 0,42
24	8,55 ± 0,92	7,80 ± 0,14	8,85 ± 1,06
48	0,92 ± 0,28	7,65 ± 0,21	7,80 ± 0,14



**Figure 1:** Antimicrobial Activity Against *Eschericia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* after 24 and 48 Hours of Fermentation

For the antimicrobial inhibitory activity, all of the samples showed the production of inhibitory zone around the well. Fresh bitter gourd juice (without the addition of lactic acid bacteria inoculum) also produced inhibitory zone diameter around 12.5-14.6 mm. From the Figure 1, the result showed that the longer fermentation time, the lower inhibitory activity of instant lactic acid bacteria was.

The ability to inhibit other microbial was from bitter gourd and also from the bacteria itself. Bitter gourd had low pH which could inhibit the growth of microbes or pathogens. Besides that, in bitter gourd, there are some functional components such as protein, alkaloids, charantin, chorine, glycosides, and goyasaponins that act as antimicrobial agent [16]. The antimicrobial effect of lactic acid bacteria is caused by the production of organic acids such as lactic acid, acetic acid, and propionic acid that can interact with the cell membrane and induce the intracellular acidification and cause protein denaturation in the other microbial [26]. From the result, different species of lactic acid bacteria had different antibacterial activity. This could be caused by the various antimicrobial substances or metabolites that produced by each lactic acid bacteria such as organic acids, hydrogen peroxide, ethanol, diacetyl, bacteriocins, etc. Among

that components, lactic acid, acetic acid, and bacteriocins were the most powerful antimicrobial agent in probiotics bacteria [2].

As shown in Figure 3, it can be seen that the antimicrobial activity of bitter gourd juice fermented with instant lactic acid bacteria was higher after 24 hours of fermentation and decreased after 48 hours of fermentation. This result was obtained because the incubation time in the research was 24 hours at 37°C and it is suitable for the production of antimicrobial agents such as lactic acid, acetic acid, or bacteriocins. Probiotic bacteria can prevent the growth of pathogens such as *Salmonella typhimurium* and *Vibrio cholerae* by competing the nutrition and attachment site on the epithelium and production of organic acids and bacteriocins [4]. In another research, they also evaluated about the ability of lactic acid bacteria in fighting against some pathogen bacteria such as *E. coli*, *L. monocytogenes*, and *S. aureus*. This research reported that all of those pathogens were inducible cellular resistance to low pH, low acids, and hydrogen peroxide [26]. Overall, among 3 different pathogen bacteria used, *Escherichia coli* and *Salmonella typhimurium* had wider inhibition zone than *Staphylococcus aureus* did. The reason of this result is that gram negative bacteria (*Escherichia coli* and *Salmonella typhimurium*) have thin peptidoglycan cell wall and susceptible toward acid metabolite [26]. Inhibitory effect of gram positive bacteria was milder than gram negative bacteria.

### 3.4 The Change of pH value and Total Soluble Solid (Expressed as Sucrose) During Fermentation

**Table 3:** The Change of pH Value and Total Soluble Solid Expressed as Sucrose Concentration During Fermentation

Fermentation (hour/s)	pH	Sucrose
Fresh Bitter Gourd (0)	5.63 ± 0.01	4.23 ± 0.06
24	3.37 ± 0.15	3.37 ± 0.15
48	3.27 ± 0.06	3.27 ± 0.06

As seen on Table 3, the initial pH value of Fresh bitter gourd juice was 5.63 ± 0.01 and this value decreased after 24 hours of fermentation, which was 4.33 ± 0.01. Results in Table 3 show that, there was also the decrease of pH value after 48 hours of fermentation in bitter gourd juice fermented by instant lactic acid bacteria. This result indicated that lactic acid (pKa = 3,86), which is the main metabolite produced by lactic acid bacteria, was produced by all the strains and kept increasing during the fermentation, thus resulted in the decreased of pH value of fermented bitter gourd juice [19].

As seen on Table 3, initial total soluble solid expressed as sucrose concentration of fresh bitter gourd was 4.23 ± 0.06. Moreover, there was the decrease of soluble solid expressed as sucrose concentration in bitter gourd juice fermented by instant lactic acid bacteria after 24 hours of fermentation. This results indicated that sucrose were metabolized by the strain, thus reduced the

total soluble solid in fermented bitter gourd juice. The decrease of total soluble solid expressed as sucrose concentration was one of the indicator of sugar consumption by lactic acid bacteria during fermentation. However, other sugars could also be utilized as carbon source by the bacteria during fermentation. The concentration of sucrose reduced significantly in comparison between 24 and 48 hours of fermentation on bitter gourd juice fermented by instant lactic acid bacteria. This results proved that metabolism of carbohydrates by *Lactobacillus* can be different in each strain, which depends on the substrate and even on the fermentation time [19]. The sugars were utilized by the bacteria, but they could not be considered as the primary sources for cell growth because of their low concentrations [19].

#### 4 CONCLUSIONS

The viability of all varieties of lactic acid bacteria in fermented bitter gourd juice increased during fermentation 24 hours and decreased after the 48 hours of fermentation. The amount of living cell was still in the minimum range of probiotic drink that was about  $10^6 - 10^7$  CFU of probiotic bacteria per milliliter. Antioxidant activity expressed as free radical scavenging activity of bitter gourd juice fermented with instant lactic acid bacteria decreased after 24 hours of fermentation and decreased further after 48 hours of fermentation. Fresh bitter gourd juice could produce bacteriocins with inhibition area about  $118.76 - 147.34 \text{ mm}^2$ , while the fermented bitter gourd juice did not produce any bacteriocins. The fermented bitter gourd juice had an antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*. The pH and total soluble solid expressed as sucrose concentration of fermented bitter gourd juice decreased gradually during fermentation. From the result, it can be concluded that bitter gourd is a suitable media for instant lactic acid bacteria growth.

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## **IMPACT OF FERMENTATION CONDITIONS ON THE EXTRACTION OF PHENOLICS AND SOME CHEMICAL PROPERTIES OF WINE FROM MANGOSTEEN PERICARP**

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### **ABSTRACT**

Phenolics are important components of wine that can be used to determine the quality of wine. Winemaking techniques and fermentation conditions are known to impact the composition. This study focuses on the impact of fermentation condition (fermentation temperature and pectinase addition) on the extraction profile of phenolics during mangosteen wine fermentation, four different treatments were performed in triplicate. The four treatments were fermentation at  $30\pm 2^{\circ}\text{C}$  with and without pectinase and fermentation at  $12\pm 2^{\circ}\text{C}$  also with and without pectinase. The temperature and pectinase addition had no significant effect on titratable acidity (as malic acid), percent alcohol but had a significant effect on pH and residual sugar ( $p<0.05$ ). Fermentation at higher temperature had lower pH than fermentation at lower temperature. The color density value was also higher for the wines fermented at lower temperature during active fermentation. However, the differences disappeared after 11 days of fermentation. Among the estimated values with the coefficient of determination ( $r^2$ ) of 0.86 of total phenolics, anthocyanin, and tannin generated using the Skogerson-Boulton model there was no significant difference among treatments except for total phenolics. Both fermentation temperature and pectinase addition affected the total phenolic concentration; higher temperature and pectinase addition resulted in higher amount of total phenolics.

**Keywords:** mangosteen wine, phenolics, fermentation conditions, pectinase addition

### **1 INTRODUCTION**

Wine is an alcoholic beverage primarily made by fermenting grapes, both red and white. However, wines can be made from different fruit species which are known as fruit wine. It is made by using the same winemaking technique as grape wine, but it is very different to grape wine in terms of flavor and mouthfeel. Tannin is one major factor that makes grape wine different from fruit wine. Most fruits do not contain high amount of tannin, which makes its wine lack of the astringency and bitterness. Most fruits also lack of anthocyanin which is the main pigment of red wine, causing the white color of fruit wine.

Mangosteen (*Garcinia mangostana*) is a tropical fruit mostly found in the Southeast Asian regions and Thailand is the largest producer and exporter. It is a round purple fruit with off-white flesh, which has a sweet and slightly acidic taste with a very pleasant aroma. Mangosteen is one such fruit that is high in tannin and could potentially produce a fruit wine that is astringent. The anthocyanin content of mangosteen also makes it a favorable fruit to ferment to wine. It contains various bioactive compounds such as phenolic acids, flavonoids, anthocyanin and xanthenes. It also has very high tannin content with the fruit shell containing about 7-13% tannin, the rind containing about 5.5% tannin and a bitter component called mangostin [1]. Apart from these bioactive compounds, mangosteen is also rich in vitamins and minerals.

The pericarp of mangosteen is abundant in phenolic compounds with most of anthocyanin in the outer pericarp (179.49 mg cyanidin-3-glucoside (cyn-3-glu) /100g) while most of total phenolic is found in the inner pericarp (3,404 mg gallic acid equivalent (GAE)/100 g) [2]. The high phenolic content of mangosteen makes it very favorable to make wine that could serve as an alternative to grape wine. For mangosteen winemaking, the tannin and anthocyanin content in mangosteen is an important factor that affect the quality of mangosteen wine including many other factors such the ratio of peel to flesh, amount and strain of yeast added, fermentation time and temperature, sugar content, etc.

Nguyen Phuoc Minh [3] studied the optimization of mangosteen wine fermentation where pectinase supplementation, water addition, yeast proliferation and yeast ratio, sugar addition and fermentation time were investigated. It was reported that pectinase enzyme at 0.15% concentration gave the best extraction recovery while water addition at ratio 20% had better sensory effect. Different fermentation batch with 19 g of sugar in 200 mL of mangosteen pulp juice was inoculated with different yeast ratios 5-11%; it was reported that 9% yeast ratio showed the most favorable sensory attribute and the highest ethanol content. 10% sugar supplementation was also ideal for fermentation and the ideal yeast proliferation time was 24 hours. To investigate the effect of fermentation time sensory analysis, ethanol concentration and sugar residue were examined. It was reported that fermentation time of 7 days resulted in the best quality wine. Other researches on various fruit wines investigated the effect of fermentation time, temperature and pH. Studies on the fermentation process of mangosteen wine is very limited and while studies on the phenolic content of grape wine are well investigated, similar studies on mangosteen wine are rare, therefore further investigation is required on the subject. Therefore, the aim of this research was to investigate the extraction profile of phenolics of mangosteen pericarp wine during active fermentation under different fermentation conditions.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Fresh mangosteens were purchased from a local market in Bangkok, Thailand. The mangosteens were washed and peeled. The mangosteen pericarp was cut into 1 cm cubes and stored at -20°C until further processing.

### 2.2 Must preparation and Fermentation

100 g of mangosteen pericarp was thawed and added into sterilized glass bottles containing 3 liters of 22-23°Brix sucrose solution with 3.3-3.5 pH.

The varied fermentation conditions was fermentation temperatures (30±2°C and 12±2°C) and the availability of added pectinase (with (w) and without (wo)). After the mangosteen pericarp was added to the sucrose solution, 0.67 g/L of commercial pectic enzyme powder (Carlson, Ohio, USA) was added to two treatments (30±2°C w/pectinase and 12±2°C w/pectinase). Prior to inoculation with *Saccharomyces cerevisiae* (Montrachet red), diammonium phosphate (DAP) was added to increase the yeast assimilable nitrogen. Four different treatments, including 30±2°C w/wo pectinase and 12±2°C w/wo pectinase were performed in triplicate. Fermentations were pressed and filtered after the process alcoholic fermentation had ceased (constant °Brix), and bottled into 750 ml glass bottles and stores at 12°C.

### 2.3 Determination of chemical compositions of mangosteen pericarp wine

The chemical compositions of the finished wine was analyzed in triplicate after alcoholic fermentation had finished. The ethanol content of wine was measured by traditional Ebulliometer (160000- Complete traditional Ebulliometer, Laboratories Dujarden-Salleron, France). The pH was measured using pH meter (pH211, Microprocessor, Hanna instruments). The titratable acidity was measured by titration with 0.1N NaOH (AOAC 926.12, 1990). The reducing sugar was measured by using the 3, 5-dinitrosalicylic acid (DNS) assay [4].

### 2.4 Color measurement and Harbertson Adams Assay

All treatments were sampled once a day at approximately 24-hour interval. 15 mL of each sample was vacuum filtered and subsequently frozen at -20°C for color measurement and Harbertson-Adams assay. Samples were thawed prior to analysis and Spectrophotometric measurement at 420, 520, and 620 nm was carried out for color density and hue. Color density was quantified as the sum of absorbance at 420, 520, and 620 nm. Hue was calculated as the ratio between absorbance at 420 and 520 nm. The predicted Adam-Harbertson values for total phenolic, tannin, and anthocyanin with the coefficient of determination ( $r^2$ ) of 0.86 was generated for all samples during active alcoholic fermentation and finished wine by using the Skogerson-Boulton model [5]. All analysis was performed in triplicate.

## 2.5 Statistical Analysis

Statistical analysis was performed by using Statistical Analysis System (SAS) program, version 9.4 (SAS Institute, Cary, NC, USA). Randomized Complete Block Design along with Fisher's least significant difference were used to discriminate the means of the chemical composition of finished wines between all fermentation treatments. All fermentation treatments and chemical analysis were performed in triplicate.

## 3 RESULTS AND DISCUSSION

The chemical composition of the wines with different treatments (fermentation temperature and pectinase addition/absence) were determined at the time of bottling (Table 1). The results indicated that different temperature and the presence of pectinase enzyme had no effect on the alcohol content and titratable acidity (TA) as malic acid. The result also indicated that fermentation at lower temperature significantly effected on the pH of the wine; lower temperature resulting higher pH value in wine.

It also shows that both temperature and enzyme availability significantly affect the amount of reducing sugar. In the wines, the reducing sugar ranged of 9–13 g/L indicated that the wine is off-dry wine.

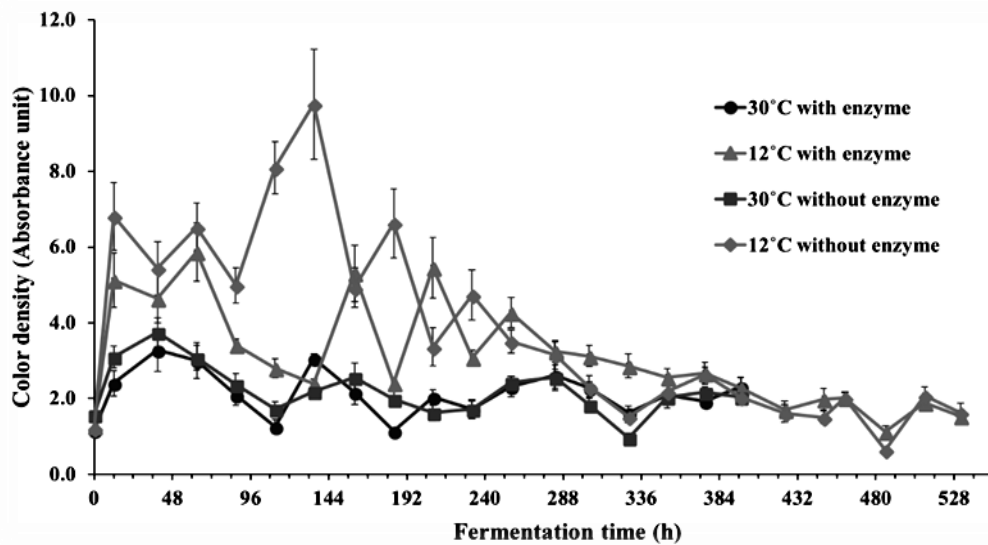
**Table 4:** The chemical compositions of finished mangosteen pericarp wines made with different winemaking conditions at the time of bottling

Level of Treatment	%Alcohol	pH	TA (g/100 mL)	Sugar (g/L)
30°C with enzyme	12.15 ± 0.17 a	3.27 ± 0.06 b	0.34 ± 0.01 a	9.77 ± 2.41 b
30°C without enzyme	12.13 ± 0.15 a	3.26 ± 0.03 b	0.33 ± 0.01 a	13.66 ± 0.66 a
12°C with enzyme	12.12 ± 0.26 a	3.36 ± 0.04 a	0.32 ± 0.06 a	13.85 ± 0.62 a
12°C without enzyme	12.36 ± 0.07 a	3.36 ± 0.02 a	0.34 ± 0.01 a	12.31 ± 1.75 ab

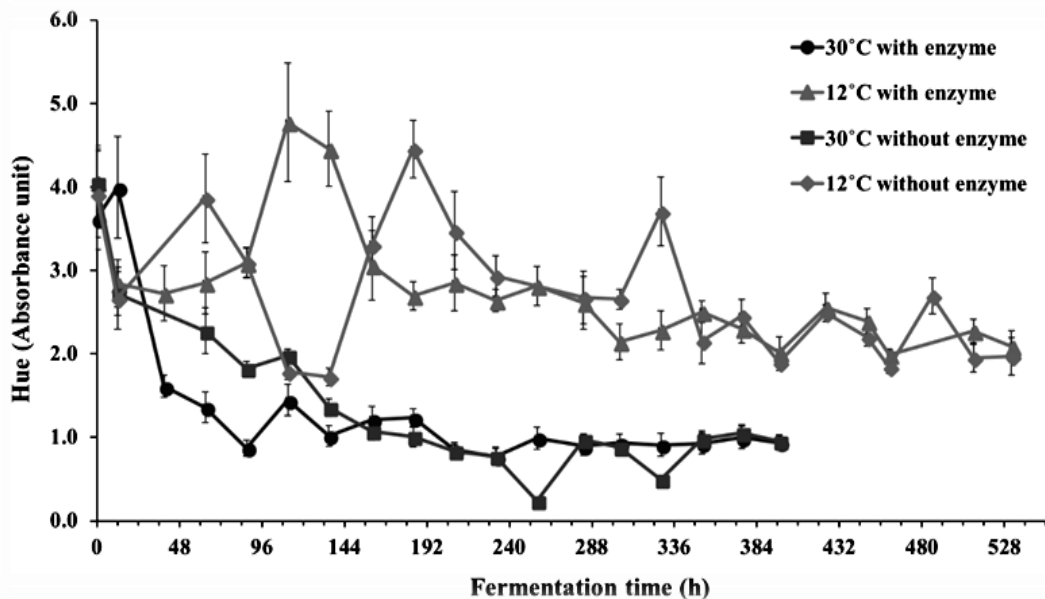
*Note: Results are expressed as mean ± standard deviation. The same letter in the same column indicates that there is no significant difference ( $p < 0.05$ ,  $n = 3$ ).*

Figure 1 shows the changes in color density for all treatments increased from the beginning and decreases from day three until the end of fermentation except the sample fermented at 12°C without pectinase. Treatments fermented at 30°C had a low color density values compared to treatments fermented at 12°C. However, these differences disappeared after 11 days of fermentation. Addition of pectinase at 30°C did not affect the color of the wine. However, at 12°C there was a slight difference between the treatments. The treatment without enzyme had higher values of color density. The color of the wine is highly dependent on the amount of pigments presenting in the fruit as well the ease of extraction [6]. Changes in the hue values (Figure 2) was opposite to that of color density (Figure 1) during fermentation for all treatments.

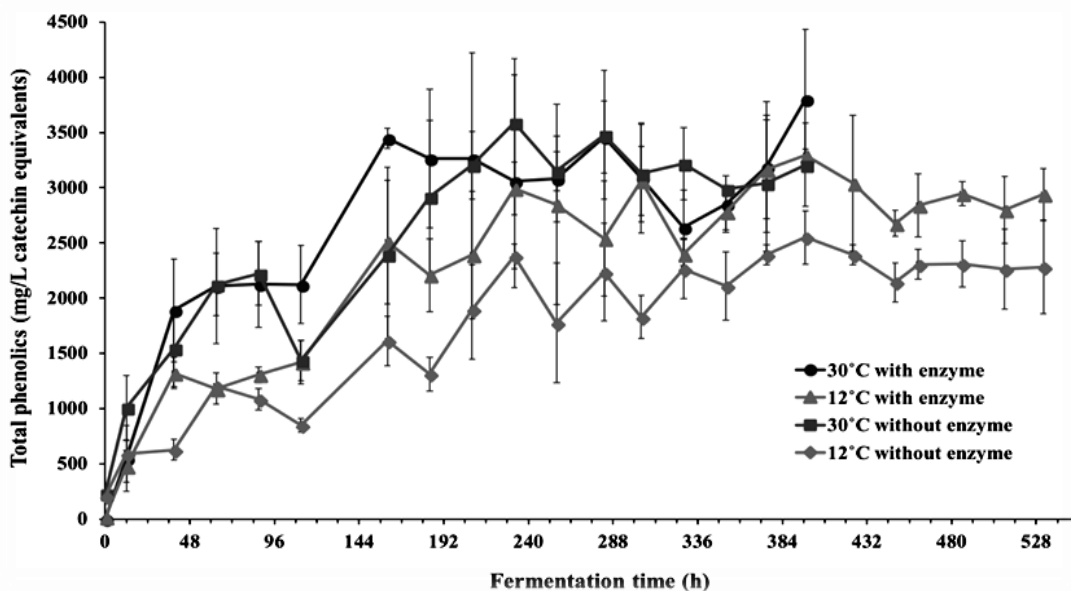
During active fermentation, lower hue values were found in the treatments fermented at 30°C compared to the treatments fermented at 12°C. This can indicate that, at lower temperature there is more extraction of yellow pigments or less extraction of red pigments compared to higher temperatures. Addition of pectinase did not have a distinct effect on the hue values.



**Figure 2:** The evolution of color density of mangosteen pericarp wines made with different winemaking conditions during active fermentation as determined by using spectrophotometer (n=3)



**Figure 3:** The evolution of hue of mangosteen pericarp wines made with different winemaking conditions during active fermentation as determined by using spectrophotometer (n=3)



**Figure 4:** Evolution of total phenolics concentration of mangosteen pericarp wines made with different winemaking conditions during active fermentation

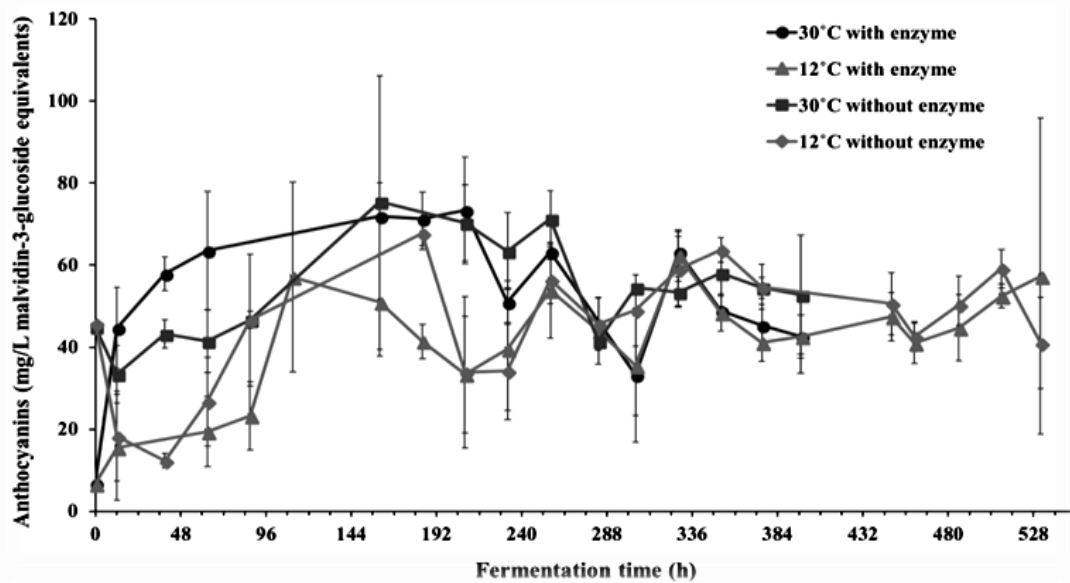
The extraction of total phenolics during active fermentation were estimated by spectrophotometer using the Skogerson-Boulton correlation model (Figure 3).

Fermentation at 30°C resulted in higher extraction of phenolic compounds compared to fermentation at lower temperature. The addition of pectinase also resulted in higher extraction at both temperatures. This is in agreement with the results found by Romero, et al. [6], higher phenolic content in grape wine was produced with added enzyme. Pectinase hydrolyzes pectin which is located between the primary and secondary cell-wall. Pectin helps hold the cell together. In the presence of pectinase, pectin is hydrolyzed and the cells are breakdown thereby, the extraction of anthocyanin and tannin increased, which leads to overall improvement in the color of the wine. Fermentation at higher temperature has been reported to increase phenolic extraction [7]. Processing temperature plays an important role and influences the extraction because temperature affects the permeability of the grape cells and the membrane [8].

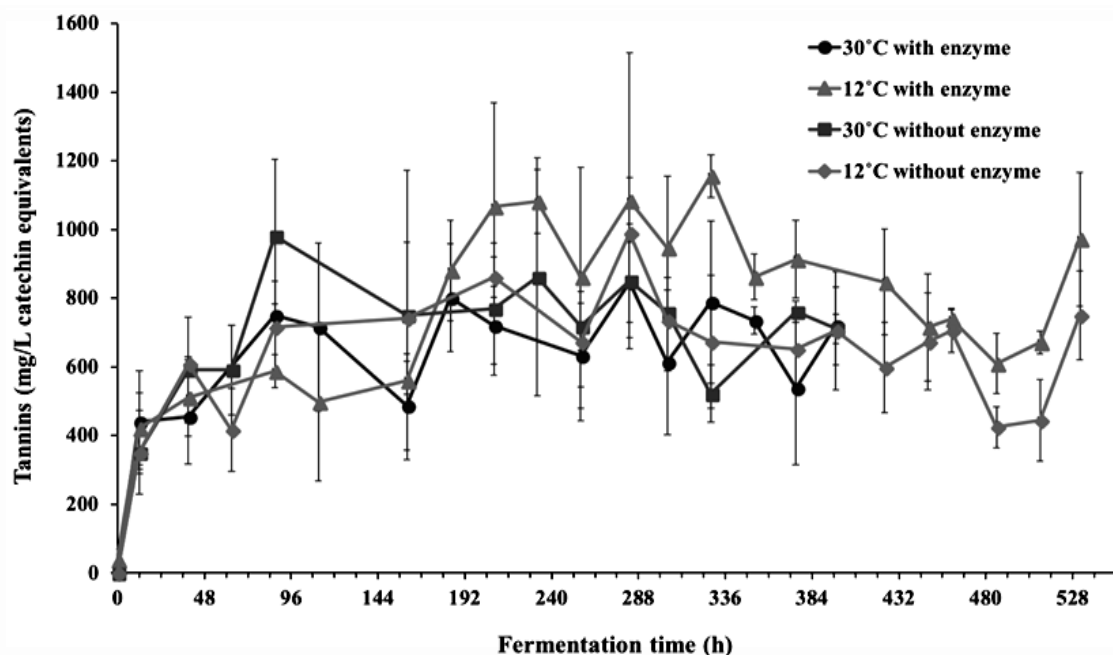
The extraction of anthocyanin during active fermentation were estimated by spectrophotometric measurement using the Skogerson-Boulton correlation model (Figure 4). The content of anthocyanin of extraction increase rapidly during the first 7-10 days' fermentation time after which, the amount of anthocyanin decreases. This is in agreement to the study conducted by Damijanić, et al. [9], where the same trend was reported in Teran wine. In case of effect of temperature, fermentation at 30°C had higher concentration of anthocyanin compared to 12°C during the first 10 days of the fermentation period. However, these differences disappeared after 11 days. The amount of anthocyanin, their localization in the grape berry, processing duration and temperature influence the rate and extent of anthocyanin extraction [10]. In theory, the anthocyanin content of the wine increases in the beginning of the fermentation until reaching a maximum value and after that the anthocyanin content slowly decreases until the end of



fermentation [11]. When the anthocyanin content of the wine and the grape has reached an equilibrium, no more anthocyanin can be extracted into the wine [12]. This might suggest that the same phenomenon occurred during the fermentation of mangosteen wine. A study on the anthocyanin of Pinot noir also reported that at 15°C and 30°C, there was little difference in the anthocyanin content [13].



**Figure 5:** Evolution of anthocyanin concentration of mangosteen pericarp wines made with different winemaking conditions during active fermentation as determined by the Skogerson-Boulton model (n=3)



**Figure 6:** Evolution of tannin concentration of mangosteen pericarp wines made with different winemaking conditions during active fermentation as determined by the Skogerson-Boulton model (n=3)

The addition of enzyme had no effect during fermentation at 12°C but at 30°C, there was more anthocyanin are extracted during the first 5 days. This indicates that temperature affects the



anthocyanin extraction only during the beginning of the fermentation while enzyme does not have much effect on the extraction.

The extraction of tannins during active fermentation were estimated by spectrophotometric measures using the Skogerson-Boulton correlation model (Figure 5). There were no significant differences in tannin concentration among treatments. This indicate that temperature and enzyme addition had no effect on tannin extraction during fermentation. The extraction profile as seen in Figure 5 shows a steady increase during the first week of the fermentation period after which it starts decreasing for all treatments. Presence of anthocyanin increases the solubility and retention of tannins [14], (anthocyanin and tannin) polymerize to form polymeric pigment [15]. This might explain the reduction in the tannin content towards the end of the fermentation period.

#### **4 CONCLUSIONS**

In conclusion, the temperature and enzyme addition had no significant effect on acidity, alcohol content but had an effect significantly on pH and reducing sugar ( $p < 0.05$ ). Fermentation at higher temperature had lower pH than fermentation at 12°C. The color density values were also higher for the wines fermented at 12°C during active fermentation. However, the differences disappeared after 11 days of fermentation. Among the estimated values with the coefficient of determination ( $r^2$ ) of 0.86 of total phenolics, anthocyanin, and tannin generated using the Skogerson-Boulton model [3] there was no significant different among treatments except for total phenolics. Both temperature and enzyme addition affected the total phenolic concentration; higher temperature and enzyme addition resulted in higher amount of total phenolics.

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## BIOETHANOL FROM COFFEE PULP IN VIETNAM

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### ABSTRACT

Coffee pulp is the first waste product obtained during the wet processing of coffee bean. Coffee pulp represents 40% of the total weight of the coffee cherry. The coffee pulp contains 25.88% cellulose, 3.6% hemicellulose and 20.07% lignin. Coffee pulp is considered as an ideal substrate of lignocellulose biomass for microbial fermentation for the production of value-added products (especially ethanol). In this study, alkaline pretreatment of the coffee pulp with NaOH (0.2 g/g biomass) in microwave at temperature 120°C in 20 min gave the best results: 71.25% cellulose remaining, 46.11% hemicellulose removing and 76.63% lignin removing. After that, the pretreated biomass was hydrolyzed with enzyme Viscozyme Cassava C (enzyme loading was 19.27 FPU/g) at temperature 50°C and 72 hours. Under these conditions maximum reducing sugars and glucose concentration in the hydrolysate were 38.21 g/L and 30.36 g/L respectively. Then hydrolysis solution was fermented by yeast *S. cerevisiae* ( $3.10^8$  cells/mL) at 30°C for 72 h. The maximum concentration of 11.28 g/L ethanol was obtained. The result indicated that, being available in plentiful amounts and non-edible material, the coffee pulp will be a potential feedstock for bioethanol production in Vietnam.

**Keywords:** Bioethanol, coffee pulp, fermentation, lignocellulose biomass, hydrolysis, pretreatment.

### 1 INTRODUCTION

Vietnam is currently the world's largest exporter of Robusta coffee as well as the world's second-largest exporter of coffee bean after Brazil. In 2016, total production of Vietnam is about 1.636.500 tons coffee bean (USDA, Coffee: World Markets and Trade) and annually make about 450.000 tons of dried coffee pulp. This coffee pulp is mainly used as a fuel for drying fruit, coffee bean or composting and replenishing coffee trees. This usage causes serious environmental

pollution. There are many researches on the use of coffee pulp in the world, such as: Feeding and digestibility studies were conducted in concrete ponds to evaluate the use of coffee (*Coffea robusta*) pulp as partial and total replacements for yellow maize in low-cost diets for catfish [1, 2], evaluated the effect of adding coffee husks to animal feed as a substitute for a mixture of corn grain, husks and cobs. Among them, researches are more concerned with producing ethanol from coffee pulp using chemical methods [3]. However, these methods have huge limitation attributed to the use of acid and alkali for hydrolysis of coffee pulp which lead to seriously environmental pollution and the use of costly and hardly manufactured equipment. Moreover, coffee pulp has a high concentration of carbohydrates and thus can be viewed as a potential raw material for bio-ethanol production [4]. Besides, recent studies indicate the excellent potential of residue utilization for bio-ethanol production, given that it does not involve costs related to raw material growth. Furthermore, it is estimated that ethanol production from agricultural residues could increase in 16 times the current production [5].

Recognizing the high potential in large quantities of coffee pulp in Vietnam, as well as in line with current global trends of finding alternative green energy for fossil fuels which have been gradually depleted by time to solve the problem of environmental pollution and climate change, this study is conducted to find a good solution to the aforesaid problem.

## **2 MATERIALS AND METHODS**

### **2.1 Materials**

Robusta coffee pulp was collected at PongDrang commune, KrongBuk district, Dak Lak province. Berries ripen has bright red, not crushed, not mold. After harvesting, the pulp was removed and dried at 65°C until 5-8% moisture content. Then pulp was crushed and sieved to obtain powder form of 0.5-1 mm. This powder was used for the entire experiment in this study.

### **2.2 Analytical methods**

The moisture content and some other compositions were analyzed according to AOAC. [6]

Total sugars (TRS) was estimated by phenol sulphuric acid method using maltose as standard. [7]

Reducing sugars (RS) in hydrolysate were measured using DNS method adapted from Miller. [8]

Monomeric sugars (glucose) was determined by using the blood glucose meter Clever Check (model TD 4230, Germany).

Cellulose, hemicellulose and lignin contents were determined by crude fiber analysis. [9]

Ethanol concentration was determined by using a Genesys UV-Vis Spectrometer (Genesis 10S). [10]

### 2.3 Pretreatment method

50 g each of dried coffee pulp was treated with 500 mL of sodium hydroxide solution (0.2 g NaOH/g biomass) and then subjected to pretreatment at 120°C for 20 min in microwave. The pretreated biomass was recovered by filtration and washed with 1000 mL of hot water (70°C) to remove remaining lignin and alkaline substances according to the method reported by (Chen et al., 2007). After washing, the pretreated residue was pressed to remove excess water and dried at 65°C until 5-8% moisture content.

### 2.4 Hydrolysis method (enzyme loading)

The commercially available enzyme Viscozyme Cassava C (Novozymes A/S, Denmark) was selected and its activity was determined using methodology proposed by Ghose (1987) and reported as filter paper units (FPU) per milliliter of original enzyme solution. The enzyme activity was measured to be 57.82 FPU/mL in this instance. This value was then used to calculate the enzyme dosage (5 mL of enzyme preparation) for the hydrolysis experiments as 19.27 FPU/g of solid fraction of coffee pulp on a wet weight basis, which is within the range that is commonly used (7–33 FPU/g substrate) [11].

For the hydrolysis experiments, 5 mL of enzyme preparation, 150 mL of 0.05 mol/L citrate buffer (pH 4.8), and 15 g (equivalent to 10%, w/v of dry material per 100 mL of solution) of pressed pretreated pulp (after dried) were added to 250 mL Erlenmeyer flasks. The containers were incubated in a thermal shaker at 50°C and 150 rpm for 72 h and then the material from each treatment was centrifuged at 2,500g for 10 min [12]. The supernatant was removed for determination of RSs, total reducing sugars (TRSs), and glucose concentrations. Control sample are the sample which are not treated with heat and alkaline.

The yield from the enzymatic hydrolysis process was calculated using the following equation. This yield takes into account only the cellulose present in the pretreated coffee pulp:

$$\text{YEH}(\%) = \frac{0,9(G_e - G_w)}{C_p} \times 100 \quad [13] \quad (1)$$

Where  $G_e$  is the glucose concentration at the end of the enzymatic hydrolysis (g glucose/L),  $G_w$  is the glucose concentration without enzyme treatment (g glucose/L), and  $C_p$  is the cellulose concentration in the pretreated material (g cellulose/L of medium to be hydrolyzed).

### 2.5 Fermentation method

The solution after hydrolysis will be divided into equal portions of 250 mL each and taken in a 500 mL Erlen flask. Then supplemented with ammonium sulfate  $(\text{NH}_4)_2\text{SO}_4$  (1 g/L), dibasic potassium phosphate  $\text{K}_2\text{HPO}_4$  (0.1 g/L), and magnesium sulfate heptahydrate (0.2 g/L), and the medium was autoclaved at 121°C for 20 min and allowed to cool at room temperature. Fermentation was carried out in 250 mL Erlen flask with  $3.10^8$  (Cells/mL) yeast *S. cerevisiae*

(which was isolated from an ethanol plant, stored in lyophilized form, and obtained from the collection of the Laboratory of the Food Technology Department at the Industrial University of Ho Chi Minh City) at incubation temperature of 30°C at 120 rpm and pH of 5 [14, 15]. Ethanol concentration was analyzed by using a Genesys UV-Vis Spectrometer at different fermentation times.

The ethanol yield was calculated as produced ethanol divided by consumed using the following equation:

$$Y_{P/S} = EC / (Gb - Ge) \quad [13] \quad (2)$$

EC (g/L) = ethanol concentration at the end of fermentation, Gb (g/L) is glucose concentration at the beginning of the fermentation, and Ge (g/L) is glucose concentration at the end of the fermentation.

Percentage of theoretical Ethanol yield:

$$Y_{et} (\%) = \frac{Y_{P/S}}{0.51} \times 100 \quad [16] \quad (3)$$

0.51 is the maximum theoretical ethanol yield when converted 1g glucose to ethanol.

## 2.6 Statistical analysis

All treatments in this study were conducted in triplicate and a 95% confidence level was applied for data analysis. ANOVA was used for one-way analysis of variance and Statgraphics software (Centurion XV) was used to determine the statistical differences between treatments.

## 3 RESULTS AND DISCUSSION

### 3.1 Characterization of solid fraction of coffee pulp

Table 1 shows that the cellulose and lignin content in coffee pulp (*Robusta coffee*) was 25.88% and 20.07% respectively. These value were higher than those found by Bonilla et al., (2014), Braham & Bressani, (1979), Menezes et al., (2014), but the hemicellulose content was similar. The difference is due to the materials used in the previous studies as Arabica coffee, not Robusta coffee as in this study.

**Table 1:** Chemical composition of coffee pulp (g/100g dry basis)

Components	This study	a	b	c	d	Sticky coffee husk <sup>e</sup>
Moisture content	73.85	-	-	77.9	82	15
Total sugars	9.18	9.70	-		-	28.7
Reducing sugars	8.34	9.63	12.40		-	24.25
Starch	10.20	-	-		-	-

Components	This study	a	b	c	d	Sticky coffee husk <sup>e</sup>
Pectin	4.37	11.37	6.50		-	-
Protein	9.52	10.47	10.1		-	7
Cellulose	25.88	20.7	17.7	23	20.6	16
Hemicellulose	3.60	3.60	2.30	20	17.2	11
Lignin	20.07	14.30	17.5	22	15.5	9
Lipid	1.22	1.20	-		-	0.3
Ash	6.29	7.33	8.30	15.4	7.9	5.4
Caffeine	0.78	-	1.3		-	1
Pholyphenols	8.69	-	1.8-8.56		-	5

<sup>a</sup> [17]; <sup>b</sup> [18]; <sup>c</sup> [19]; <sup>d</sup> [13]; <sup>e</sup> [20]

Coffee husks and pulp are comprised of the outer skin and attached residual pulp, and these solid residues are obtained after de-hulling of the coffee cherries during dry or wet processing, respectively [21]. In that, the coffee pulp only included outer skin and fruit pulp. The sticky coffee husk included skin, fruit pulp and maybe a little pectin and parchment. So the total sugars (28.7%), reducing sugar (24.25%) were more than the coffee pulp (9.7%, 9.63%).

**Table 2:** Comparison of composition of Lignocellulosic Feed Materials

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Reference
Corn fiber	14.28	16.8	8.4	[22]
Rice husk	24.3	27.22	12.59	[23]
Corn stalk	34.51	24.87	14	[23]
Wheat straw	38.2	21.2	23.4	[24]
Switch grass	31.0	20.4	17.6	[24]
Corn cobs	45	35	15	[11]
Hard wood	50	23	22	[11]
Bagasse	38	27	20	[25]
Coffee pulp	25.88	3.6	20.07	This study



According to Palonen and Hetti, lignocellulose biomass is a major structural component of woody plants and other plants such as grass, rice and maize. The major constituents of lignocellulose are cellulose, hemicellulose, and lignin [26]. The crude fiber in coffee pulp included: cellulose 25.88% (equivalent to 52.23%, g cellulose/100g crude fiber), hemicellulose 3.6% (equivalent to 7.3%, g hemicellulose/100g crude fiber) and lignin 20.07% (equivalent to 40.5%, g lignin/100g crude fiber). It has a similar proportion of lignocellulose with other sources, such as: corn fiber or rice husk (Table 2). The typical proportion of cellulose, hemicellulose and lignin in lignocellulose biomass included: cellulose (40-60%), hemicellulose (20-40%) and lignin (10-25%) [27]. Coffee pulp, therefore, are also considered a source of lignocellulose biomass which can be used in the production of bioethanol (second generation ethanol production).

### 3.2 Enzymatic hydrolysis

The coffee pulp was alkaline pretreated with NaOH (0.2 g/g biomass) at 120°C for 20 min in microwave before hydrolysis. The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials. Of these methods, alkaline pretreatment method are more efficient for materials rich in lignin [11].

To assess the effectiveness of alkaline pre-treatment, the percentage of hemicellulose and lignin removed and the enzyme's ability to hydrolyze to produce more or less glucose must be taken into account. In addition, to evaluate the effectiveness of the pre-treatment process, the control sample was established. Control samples (without pretreatment with alkali) were also hydrolyzed at the same time with the test sample under the same conditions. However, the hydrolysis effect varies greatly (Table 3).

**Table 3:** Concentration of Reducing Sugars (RS), Total Reducing Sugars (TRS) and Glucose after enzymatic hydrolysis

Test	TRS (g/L)	RS (g/L)	Glucose (g/L)	Reference
Coffee pulp ( <i>Robusta</i> )	48.2 <sup>a</sup> ± 0.18	38.21 <sup>a</sup> ± 0.22	30.36 <sup>a</sup> ± 0.59	This study
Control	15.8 <sup>b</sup> ± 0.46	14.15 <sup>b</sup> ± 0.39	12.63 <sup>b</sup> ± 0.61	This study
Coffee pulp ( <i>Arabica</i> )	66.15	38.13	27.02	[13]
Wheat straw	-	-	279*	[28]
Bagasse cane	-	-	21	[29]

\* mg/g biomass



*Various lowercase letters in the same column denote significant difference ( $p < 5\%$ )*

Table 3 shows that YEH (yield of enzymatic hydrolysis) was 76.8% (calculated from formula 1). It means that 76.8% of cellulose is converted to glucose after hydrolysis. The productivity is relatively high. According to Menezes et al. (2014), hydrolysis of coffee pulp (Arabica) resulted in producing the glucose amount of 27.02 g/L and hydrolysis efficiency of 60.48%. This productivity is lower than that in this study. This is explained as follows: when pretreatment of lignocellulose by alkaline was combined with microwave hydrolysis, the hydrolysis yield was higher and the hydrolyzed sugar content was improved compared to alkaline pre-treatment. In addition, the alkaline pretreatment combined with microwaves increases the surface area of cellulose from the breakdown of the hemicellulose and lignin layers thereby increasing the sensitivity of hydrolysis enzymes [29]. On the other hand, Chen et al (2007) used barley straw pretreated with 2% NaOH at 120<sup>0</sup>C and found 74.03 to 84.89% conversion of cellulose to glucose during enzymatic hydrolysis, using cellulase concentration equal from 40 to 60 FPU/g cellulose supplemented with cellobiase (Novozyme 188), a result significantly better than that obtained for this study with an enzyme loading of 19.27 FPU/g of substrate. [30]

The productivity increased considerably when the amount of enzyme was increased (FPU/g) or enzyme cellobiase was supplemented (CBU/g). According to Chen et al (2008), the hydrolysis of corn straw pretreated with 2% NaOH for 1 h at 80<sup>0</sup>C using 8% concentration of substrate and 20 FPU cellulase/g substrate (which contains in its mixing 1.64 CBU/g) after 60 h produced a RS concentration equal to 52 g/L and high amount of cellobiose. When increased activity of cellobiase to 10 CBU/g substrate, the RSs content was 64.1 g/L at the end of the hydrolysis [31]. This was explained by Sun and Cheng (2002) as bellows: cellulase activity is inhibited by cellobiose, and glucose to a lesser extent, and these problems can be solved by the addition of cellobiase. In the hydrolysis of lignocellulosic biomass, cellulases attack the cellulose chain to form glucose and cellobiose, the latter then being decomposed to glucose by cellobiase. Thus, the presence of cellobiase is important in reducing the inhibition of cellulase by cellobiose for obtaining a high yield of sugars.

### **3.3 Fermentation**

The glucose concentration present in the hydrolysate was consumed by the yeast, starting with an initial concentration of 36.58 g/L and reaching 6.1 g/L at the end of fermentation after 72 h. The final concentration of ethanol was 11.28 g/L with a yield of 0.37 g ethanol/g glucose. The Control (un-pretreated sample hydrolyzed and fermented in the same conditions with the study samples) showed initial glucose concentration of 20.85 g/L and 4.17 g/L at the end of fermentation. Ethanol concentration was only 5.3 g/L (table 4). Therefore, it was necessary to have the pretreatment process to remove lignin and hemicellulose.

**Table 4:** Glucose and Ethanol Contents, Ethanol Yield (Yp/s)

Fermentation Time (h)	Glucose (g Glucose/L)	Ethanol (g Et/L)	Yp/s (g Et/g Glucose)	Ethanol yield Yet (%)
0	36.58 <sup>*,a</sup> ± 0.54			
24	9.54 <sup>b</sup> ± 0.49	7.11 <sup>a</sup> ± 0.32	0.27	51.56
48	7.42 <sup>c</sup> ± 0.42	10.74 <sup>b</sup> ± 0.41	0.36	72.2
72	6.1 <sup>d</sup> ± 0.36	11.28 <sup>c</sup> ± 0.31	0.37	72.55
72 (Control)	4.17 <sup>e</sup> ± 0.27	5.3 <sup>d</sup> ± 0.32	0.31	60.7
96	5.55 <sup>f</sup> ± 0.4	11.13 <sup>c</sup> ± 0.28	0.35	70.3

\*Including the glucose concentration (g/L) in hydrolysis solution and in yeast culture supplemented during the fermentation.

Various lowercase letters in the same column denote significant difference ( $p < 5\%$ )

According to the study of Menezes et al (2014), the coffee pulp (*Arabica*) was pretreated by 4% (w/v) NaOH (equivalent to 0.2 g/g biomass) then was hydrolyzed with 13.82 FPU/g of cellulase. 27.02 g glucose/L and 11.99 g ethanol/L was obtained after hydrolysis and fermentation (table 5).

In this study, pretreatment process was coupled with microwave. As a result, the hydrolysis efficiency was higher with 30.36 g of glucose/L formed. However, ethanol yield from fermentation was only 11.28 g/L, which was slightly lower than that of the study by Menezes. This may be due to the yeast strain used. In the study of Menezes, dry yeast (3 g/L) was used, while in this study yeast was used as a secondary breed and then added to hydrolysate with a cell density of  $3.10^8$  (cells/mL). It was also possible that the yeast isolation and culture process was not good, so the fermentation efficiency was low, only 0.37 (compared to 0.51 in theory, 1 g of glucose will produce 0.51 g ethanol through fermentation). In study of Chen et al (2007), barley straw was pretreated with 2% sodium hydroxide at 121<sup>0</sup>C for 60 min. The pretreated material has undergone enzymatic hydrolysis with celluclast 1.5L at a concentration of 40 FPU/g glucose and Novozyme 188 (cellobiase). The hydrolysate was inoculated with *S. cerevisiae* (ATCC 24859) and incubated at 30<sup>0</sup>C for 72 h. At the end of the fermentation the ethanol yield obtained (Yp/s) was 0.31 g ethanol/g glucose, lower than the value found in this study (0.37).

**Table 5:** Comparison of literature data on ethanol production from lignocellulose biomass

Lignocellulose biomass	Ethanol production	Yp/s (g Et/g Glucose)	Reference
Sticky coffee husks	13.6 g/L (8.49 g/100g)	0.38	[20]
Corn stalks	5 g/L	0.44	[32]
Barley straw	10 g/L	0.44	[32]
Sweet sorghum bagasse	16.2 g/L	0.31	[16]
Wheat straw	18.1 g/L	0.32	[16]
Corn stover	16.8 g/L	0.33	[33]
Coffee pulp (Arabica)	11.99 g/L	0.4	[13]
Coffee husk	7.9 g/L	-	[34]
Coffee pulp (Robusta)	11.28 (11.36 g/100g)	0.37	This study

Kefale et al (2012) hydrolyzed coffee pulp using sulfuric acid concentration of 1, 2 and 4% for 1 hour, the obtained ethanol concentration were 6.097, 4.395 and 3.323 g/L, respectively [35]. Dilute acid hydrolysis resulted in low ethanol production when compared with distilled water hydrolysis. The maximum ethanol concentration of 6.315 g/L was obtained from coffee pulp hydrolyzed with distilled water. Martin et al (2002) fermented sugar cane bagasse pretreated with steam explosion and hydrolyzed with cellulase (26 g RS/L in hydrolysate) and final concentrations of ethanol equal to 7.4 g/L (0.28 g ethanol/g RS) and 8.2 g/L (0.31 g ethanol/g RS) were achieved using the yeasts *S. cerevisiae* ATCC96581 and *S. cerevisiae* TMB3001, respectively [29].

150 mL of filtrate yeast (*Saccharomyces creviciae*) was added at a concentration of 5.0 g/L concentration and subjected to fermentation for 48 h at 30°C in a shaker incubator at 120 rpm. Ethanol yield in the fermented broth was found to be 0.5, 0.46 and 0.46 g/g of sugar in squeezed CAP, DCP and WCP. Theoretical ethanol yield (Y<sub>max</sub> %) of squeezed CAP, DCP and WCP was found to be 46, 9.35 and 40% respectively [3].

Other authors (table 5) obtained after fermentation of hydrolyzed barley straw a final ethanol concentration equal to 10 g/L [32]. Gouvea et al (2008) fermented whole coffee husk in water (13%, w/v) with commercial Baker's yeast *S. cerevisiae* and achieved a final ethanol concentration equal to 13.6 g/L [20].

The higher conversion efficiency of glucose to ethanol and the low ethanol is attributed to low concentration of glucose in hydrolysis solution and poor pre-treatment process or low cellulose content in the raw material. This is consistent with a study by Belkacemi et al. (2002), with an ethanol conversion efficiency of 0.44 and the produced ethanol of only 5 g/L (table 5). However, the results from a study by Ballesteros et al (2004) are opposite. Conversion efficiency reached only 0.32 while 18.1 g/L ethanol were formed (Table 5). Thereby, it was found that the conversion efficiency of ethanol indicated that the glucose content in the fermentation broth was high. The high glucose content showed that the hydrolysis process was very effective or the fermentation process was very good.

#### 4 CONCLUSIONS

One hundred gram of dry coffee pulp produced 11.36 g ethanol (corresponding to glucose conversion efficiency of ethanol 0.37). Comparing with the literature data it can be seen that ethanol production by fermentation of a solid fraction of coffee pulp is quite potential. Moreover, there are probabilities to improve ethanol yields in this study, including the use of a combination of yeast strains for xylose fermentation, hydrolysis coupled with concurrent fermentation, or selection of the more potent strains of *S. cerevisiae*.

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## EFFECTS OF STORAGE TEMPERATURE ON THE PHYSICAL AND CHEMICAL PROPERTIES OF BROCCOLI

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### ABSTRACT

Broccoli (*Brassica oleracea* var. *italica*) is a perishable vegetable which has short storage time. Therefore, application of postharvest techniques is needed to protect its marketability. This study investigated effects of storage temperature on physical (appearance, overall change of color, weight loss, total soluble solids, firmness) and chemical properties (L- ascorbic acid, titrate acidity, chlorophyll content, antioxidant capacity and total phenolic content). Broccoli stored at 2°C remained most attributes analyzed during 15-days. Storing the floret at room temperature dramatically reduced its appearance after 5 days. The samples stored at 5°C had comparable results with those stored at 2°C after 20 days, except the appearance score.

**Key words:** Broccoli, storage, temperature, antioxidant capacity, total phenolic compounds

### 1 INTRODUCTION

Broccoli, *Brassica oleraceae* var. *italic*, has brought benefits for human health such as protecting human against DNA damage, boosting liver function, promoting iron absorption, lowering cancer survival [1]. It has been produced worldwide in which Vietnam has produced 98,819 tons per year, ranking at 18<sup>th</sup> [2].

Temperature is one of important factors affecting the storage life of vegetables. At the optimum temperature, the aging of vegetables, softening, undesired textural and color changes, moisture loss, and losses due to pathogen invasion can be delayed [3]. According to the Institute of Food Science and Technology, shelf life is the period of time in which the remaining of food product will be safe. In details, food could be retained its desired sensory, chemical, physical, microbiological and functional characteristics when stored under recommended conditions [4].

It has been reported that broccoli has an extremely high respiration rate [3]. This makes the floret has a short shelf life of 2-3 days [5]. According to Tourte et al., [6], the higher storage temperatures applied the shorter shelf life of broccoli obtained. Thus, this is the challenge to maintain quality and extend the shelf life of the florets. The present productivity and quality of the broccoli are good; however, it does not reach the satisfaction's customers. Furthermore, based on the global consumption of broccoli statistic, Vietnam has the high opportunity to export



vegetable, especially broccoli [7]. Therefore, a study on prolonging broccoli shelf life is necessary. Recently, many researches on broccoli preservation have been reported; however, in Vietnam, there has been no study conducted yet. This research would release the data about influences of temperature on the quality of broccoli, help to reduce postharvest loss, prolong the vegetable shelf life and increase economic benefits for producers.

## **2 MATERIALS AND METHODS**

### **2.1 Sample preparation**

Fresh raw broccoli in uniform size and weight without treatments harvested at a farm of Nông Sản Sạch Lâm Đồng Company were immediately transported to Ho Chi Minh City in a day to maintain its freshness.

At the laboratory of International University, the leaves of broccoli were trimmed as treated in the supermarket and gently washed under the tap to remove debris, insect and soaked in chlorinated water (200  $\mu$ M sodium hypochlorite solution) for 2 min [8] before further treatments. Broccoli was stored and examined in 5 day interval until the quality reduced to 2 scores of appearance (Table 1).

The fresh broccolis were stored at 2°C, 5°C, 10°C and room temperature without packaging. During the storage periods, the chemical and physical properties were analyzed after each 5-day. The experiment was done in triplicate.

### **2.2 Appearance score**

Fernández-León et al. [9] described the appearance of broccoli using scale of 5-score measured after each 5-day. Broccoli was taken photograph and appearance was evaluated based on the scale presented in Table 1 in which 5 was excellent with no defects, 4 was good with minor defects, 3 was acceptable with moderate defects, 2 was poor with major defects and 1 was inedible. The evaluation of broccoli appearance was described as follows.

**Table 1:** 5-score measuring scale of broccoli appearance

<b>Score</b>	<b>Description</b>
5-excellent	Have a fresh green color, no dehydration, no decay, no fungal or bacterial rots in head and stem.
4-good	Have a fresh green color, slightly dehydrated, no decay, no fungal or bacterial rots in head and stem.
3-acceptable	Have a still green color, have small yellow plots (10-25% surface), moderate dehydrated, no decay, no fungal or bacterial rots in head and stem.
2-poor	Have a still green color, have yellow plots (>50% surface), extremely dehydrated, have decay, fungal or bacterial rots in head and stem.

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1-very poor	Have totally yellowish plots (100% surface), extremely dehydrated, have decay, fungal or bacterial rots in head and stem.
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## 2.3 Color measurement

The color analyzer (LUTRON RGB-1002) was used to measure the color of broccoli based on the HSL, in which H stands for Hue value (0 to 1.000), S stands for Saturation value (0 to 1.000) and L stands for Luminance value (0 to 1.000).

The HSL unit was transferred into LAB unit in which L stands for Lightness (0 to 100), a stands for color from Red to Green (-128 to 128), b stands for color from Blue to Yellow (-128 to 128). The overall change in color was obtained using the relationship [10]:

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Where:

$\Delta L$ ,  $\Delta a$  and  $\Delta b$  are deviations of L, a, and b values of fresh sample.

$\Delta L = L_{\text{sample}} - L_{\text{standard}}$ ;  $+\Delta L$  means sample is lighter than standard,  $-\Delta L$  means sample is darker than standard.

$\Delta a = a_{\text{sample}} - a_{\text{standard}}$ ;  $+\Delta a$  means sample is redder than standard,  $-\Delta a$  means sample is greener than standard.

$\Delta b = b_{\text{sample}} - b_{\text{standard}}$ ;  $+\Delta b$  means sample is yellower than standard,  $-\Delta b$  means sample is bluer than standard.

## 2.4 Weight loss (%)

Electronic Balances (Shimadzu TXB-622L, Japan) was used to weigh the sample before and after storage. Weight loss is expressed as a percentage

$$\text{Weight loss} = \frac{W - W_i}{W} \times 100\%$$

Where in:

W is the initial weight of the broccoli sample (g)

$W_i$  is the weight of the sample at the time of testing (g)

## 2.5 Firmness

The firmness of broccoli was measured using Fruit hardness tester (FR-5120 Lutron Electronic, Taiwan).

$$P = \frac{N}{S}$$

Where:

P: Hardness value of fruit (Kg/cm<sup>2</sup>)

N: Pressure of dynamometer (N, Kg, LB)

S: Area of pressure (cm<sup>2</sup>)

## **2.6 Chlorophyll content (mg/100g FW)**

Chlorophyll was extracted from 1.0 g broccoli samples by homogenization in 15 mL of acetone – hexane (4:6) solvent. The absorbance was then read at 663, 645, 505, and 453 nm using a spectrophotometer (Genesys 10S Thermo, USA) [11]. Total chlorophyll was calculated in mg/100g as following:

$$\text{Total chlorophyll} = 0.671 \times A_{663} + 1.6711 \times A_{645}$$

## **2.7 Total soluble solids (°Brix)**

Briefly, 50 g broccoli was homogenized and then centrifuged at 10,000 rpm for 20 min. The supernatant was collected in order to measure total soluble solids (TSS) using a refractometer (RX-5000 Atago, Japan). The result was expressed as °Brix.

## **2.8 Titrate acidity (%)**

Titrate acidity (TA) was estimated by titrating 5.0 mL extract with 0.1 N NaOH until pH = 8.2 and expressed as the percentage of citric acid, equivalent weight is 64.04 [5] with the formula in details.

$$\% \text{ acid (wt / wt)} = \frac{N \times V_1 \times E_q \text{ wt}}{V_2 \times 1000} \times 100$$

Where: N is normality of titrant, usually NaOH (m Eq./ml)

V<sub>1</sub> is volume of titrant (ml)

Eq. wt. is equivalent weight of predominant acid (mg/m Eq.)

V<sub>2</sub> is volume of sample (ml)

1000 is factor relating mg to gram (mg/g)

## **2.9 L-ascorbic acid (mg/100g FW)**

L- Ascorbic acid contents (L-AA) was determined using the titrimetric method based on AOAC [12]. Briefly, 5.0 g of fresh broccoli samples were homogenized and placed in volumetric flask to measure volume after mixing with 30 ml of 2% oxalic acid. Then, the extracted solution (10ml) was titrated with 2,6-dichloroindophenol solution. The L- ascorbic acid contents were calculated by following formula

$$X = \frac{(V - V_0) \times T}{W} \times 100$$

Where: X is the Vitamin C content in each 100 g sample (mg/100g FW)

T is the amount of ascorbic acid standard solution (mg/mL, equivalent to 1 mL of dye solution)

V is the volume of the dye solution used to titrate the filtrate (ml)

V<sub>0</sub> is the volume of the dye solution used to titrate the control (ml)

W is the amount of the sample in the tested filtrate (g)

### **2.11 Extraction of total phenolic contents**

Approximately, 0.1 g of freeze-dried broccoli was ground and homogenized with 10 ml of 80% methanol, then the homogenate was centrifuged at 2000g for 20 min. Supernatants were collected and the residues were homogenized with 10ml of 80% methanol to re-centrifuged [13]. The final extracts were stored at 4°C for further measurement.

### **2.12 Determination of total phenolic contents**

Total phenolic contents (TPC) were measured following the procedure described by Singleton and Rosi [14]. Diluted extracts (0.5 ml) were mixed with 5 ml of 2N Folin–Ciocalteu’s phenol reagent (Sigma-Aldrich, Germany) and 5.0 ml of 7.5% sodium carbonate solution. The mixed solution was vortexed and incubated at room temperature for 30 minutes, then quickly cooled and measured the absorbance at 760 nm using a UV-visible spectrophotometer (Genesys 10S Thermo, USA). Gallic acid (Sigma-Aldrich, Germany) was used to calibrate standard curve and results were expressed as gallic acid equivalents (mg GAE/100 g FW).

### **2.13 Measurement of antioxidant capacities**

Antioxidant capacities (AC) of broccoli were measured accorded to a method of Porter [13]. Approximately, 4.0 ml of 0.1 mM methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) was mixed with 0.2 ml of extract. The control was contained 4.0 ml DPPH solution and 0.2 ml of methanol 80%. The absorbance was measured using UV-visible spectrophotometer at 521 nm. The potential of scavenging capacity of the floret was measured by comparing of the reduction of color in the examined samples with the blank (solution without plant extract) using the following equation

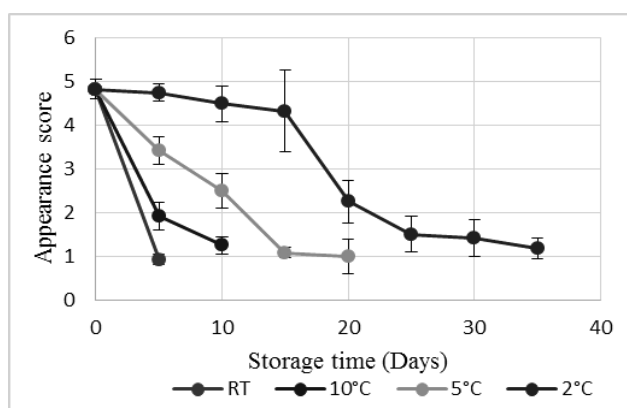
$$\% \text{ inhibition DPPH (\%)} = \frac{A \text{ control (t = 0)} - A \text{ sample (t = 3 min)}}{A \text{ control (t = 0)}}$$

## 2.14 Statistical analysis

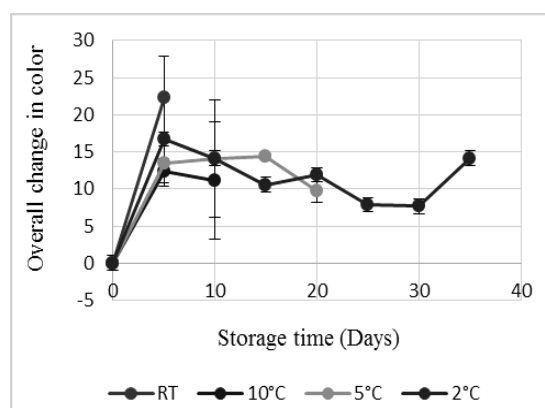
All experiments were performed in triplicate. Data are expressed as the mean values  $\pm$  standard deviation (SD) derived from triplicate determinations. The statistical analysis of the data was done by analysis of variance (ANOVA) using the software Minitab<sup>®</sup> version 16 (Minitab Inc., USA). The results were significant differences for  $p < 0.05$ .

## 3 RESULTS AND DISCUSSION

Figure 1 shows reduction trends of broccoli appearance observed at all storage temperatures. Among studied temperatures, at the same storage duration, broccoli stored at 2<sup>0</sup>C achieved the highest score, followed by those stored at 5<sup>0</sup>C, 10<sup>0</sup>C and room temperature (RT).



**Figure 1:** Effects of storage temperature on the appearance score of broccoli



**Figure 2:** Effects of storage temperature on overall change in color of broccoli

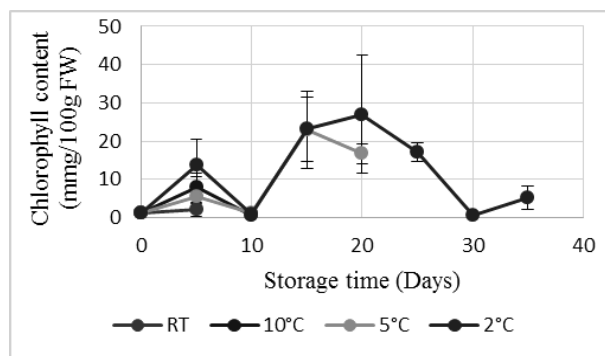
The most important changes in the visual quality attributes of broccoli during storage were associated with loss of green color and development of yellowing, which increased as storage time and temperature increased. Changes in the color of the florets were observed before occurring any major signs of wilting or loss of head compactness, particularly at temperatures higher than 5<sup>0</sup>C. At those temperatures, the color changes occurred remarkably fast and therefore, limited the postharvest life of broccoli. Broccoli stored at 2<sup>0</sup>C maintained acceptable visual quality during 20 days, with only a subtle yellowish discoloration being perceptible during 10 days of storage in some of the florets on the central part of the broccoli head.

As can be seen from Figure 2, the overall change in color of broccoli at all temperatures dramatically increased during 5 days of storage and then tend to decreased as the time extended. Moreover, broccoli stored at room temperature obtained the highest changes in the overall change in color. At room temperature, broccoli completely changed to yellow after 5 days. Broccoli stored at RT and 10<sup>0</sup>C were mature after 5 days, therefore, there was no change of color because the green color had been excreted completely. In general, color changes in broccoli during storage were associated with an increase in L\* (brightness) and chroma values and decrease in

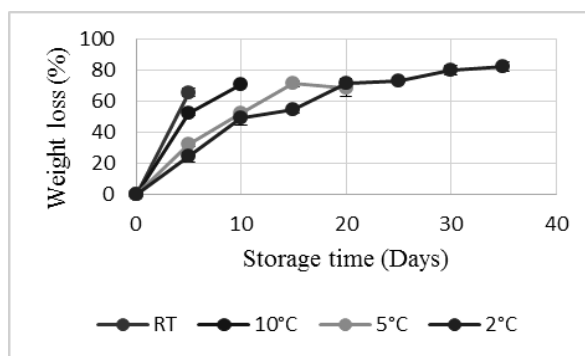
hue angle and chlorophyll content, as a result of yellowing of the florets [15]. However, the color changes of broccoli at 2°C and 5°C were not from green to yellowish color. They changed from green to dark green which showed clearly in appearance test.

Chlorophyll contents of broccoli at different temperatures fluctuated during storage (Figure 3). Change of yellowing in broccoli appearance could be explained based on the destruction of chlorophyll over time. Chlorophyll content was decreased during storage and yellow pigments appeared as carotenoids were exposed. Carotenoids coexisted with chlorophylls in the green vegetable [16]. It was reported that there was a relation of color changes between the yellowing process of broccoli and the degradation of chlorophylls [9].

On the other hand, yellowing of the florets followed major physiological changes that occurred during the post-harvest period and that contributed to broccoli senescence. Yellowing is related to phytohormones in the broccoli senescence which is combining between increased gibberellin and decreased cytokinin and auxin [17]. Moreover, ethylene, which is a plant stress hormone, can trigger the biosynthesis of enzymatic cascades involved with important events such as ripening and yellowing of broccoli. Robertson [18] reported that the ethylene production rate in broccoli is moderate-high, ranging from 3.3-27.0  $\mu\text{L/kg h}$  at 20°C. Therefore, the higher the storage temperature, the faster the color of broccoli changed yellow.



**Figure 3.** Effects of storage temperature on chlorophyll contents of broccoli



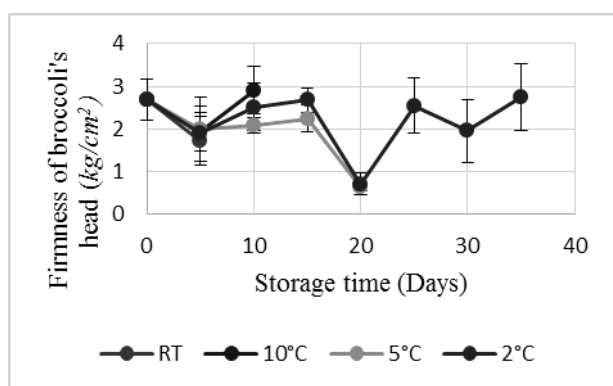
**Figure 4.** Effects of storage temperature on weight loss of broccoli

Broccoli stored at 5°C maintained acceptable quality during 20 days, yet after 5 days the head of broccoli appeared slight yellowish. After 20 days, a markedly yellowish-brown discoloration developed in some of the florets, which increased as storage progresses. Broccoli stored at 5°C maintains acceptable quality during 15 days. Yellowing of the florets was very fast in broccoli stored at 10°C and RT, and after only 5 days some of the florets developed a yellowish-green color and yellowish-gold, respectively, which increased as storage progresses.

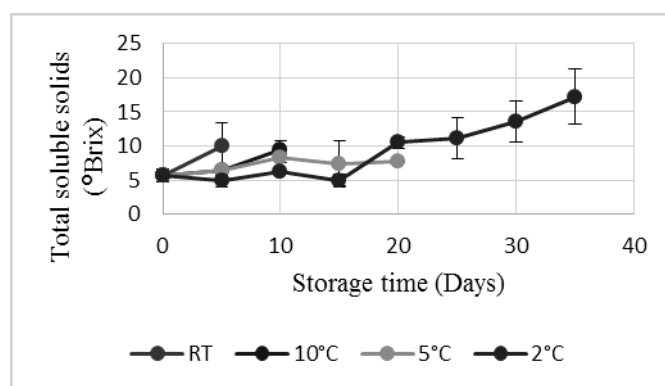
As seen from Figure 4, weight loss increased by the time. At all temperatures, moisture contents decreased dramatically by the time, however, at the same storage time, higher temperature resulted in lower moisture contents. Eskin and Robinson [3] claimed that if the water activity of food is kept constant, increasing temperature resulted in decreasing the water content. The heat

absorption provides energy for water removal from food solids. Therefore, this interprets the decreasing of moisture content. In postharvest, the vapor of water causes weight loss. The more loss of water, the lower score of appearance was observed.

Figure 5 shows that at all temperatures; there were insignificant changes of broccoli's firmness during 15 days. Firmness in vegetable or toughening is formed by lignification of the primary cell wall and formation of the secondary cell wall. In this stage, vegetable tissues start the differentiation process while the cell continues growth and enlargement which leads increasing amount of fibrous tissue. Fernández-León et al. [9] stated that weight loss and firmness values were highly correlated. They mainly related to turgidity loss and dehydration. The weight loss affects directly in the texture since tissue dehydration increases in elasticity and tissue became more fibrous and responsible for the increase in the stem shear force found during storage [19]. Therefore, the firmness increased during at storage. However, when the florets ripen, protopectins are hydrolysed to release soluble pectin, causing the softness of cell walls. As a result, the firmness is reduced. Increasing the fibrous tissue combines with increasing soluble pectin might keep the firmness of broccoli was insignificantly changed during storage.



**Figure 5.** Effects of storage temperature on firmness of broccoli head.



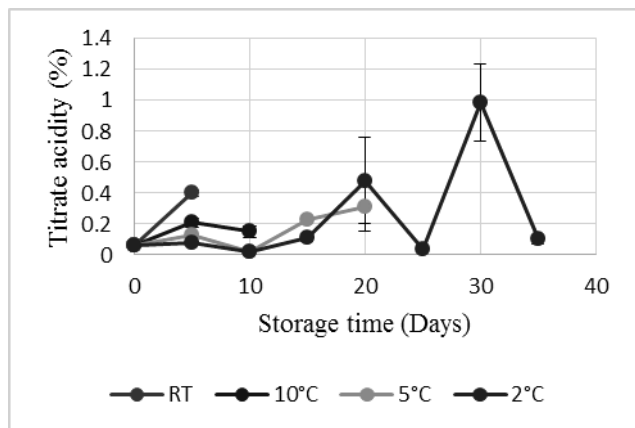
**Figure 6.** Effect of storage temperature on total soluble solids of broccoli

Figure 6 shows a rising trend of TSS at all studied temperatures. TSS at 2°C still remained with no significant difference from that at RT after 15 days of storage. During the storage periods, the total soluble solid contents were continuously increased due to hydrolysis of polysaccharides [20]. Total soluble solids can be used to determine the maturity of fruits and vegetables. As can be seen in Figure 7, titratable acidity of broccoli at 5°C, 10°C and RT increased during storage. However, titratable acidity of broccoli stored at 2°C was maintained from the initial value 0.064% to 0.10%.

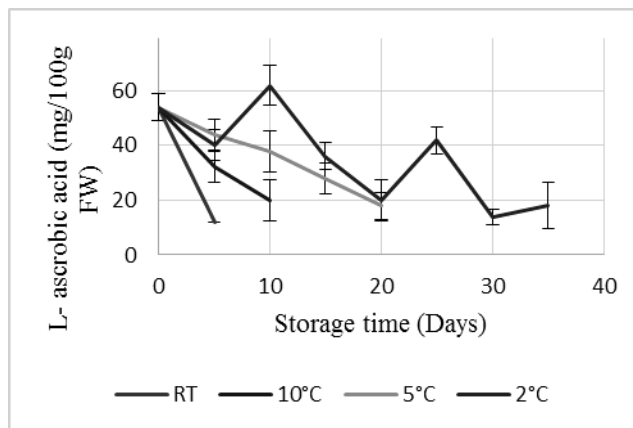
Ascorbic acid content in broccoli stored at different temperatures decreased sharply from the initial value (Figure 8). The ascorbic acid loss was related to reduce ascorbic acid reductase activity and increased lipid peroxidation during storage [16]. The altering the cellular integrity due to dehydration resulted in increasing the degradation of ascorbic acid by oxidase enzymes



[15]. On the other hand, the loss of ascorbic content of broccoli during storage could be due to its water solubility, thermic degradation and enzymatic oxidation [21].

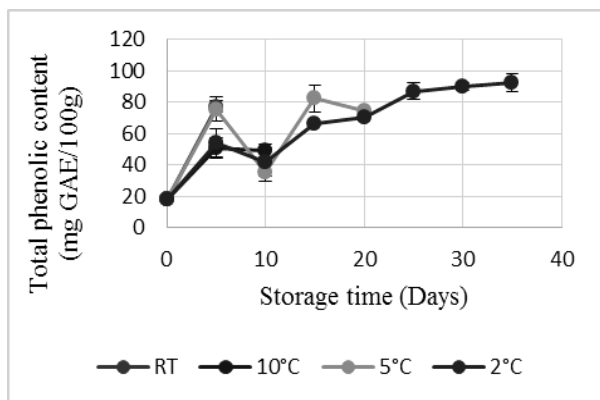


**Figure 7.** Effects of storage temperature on titratable acidity of broccoli.

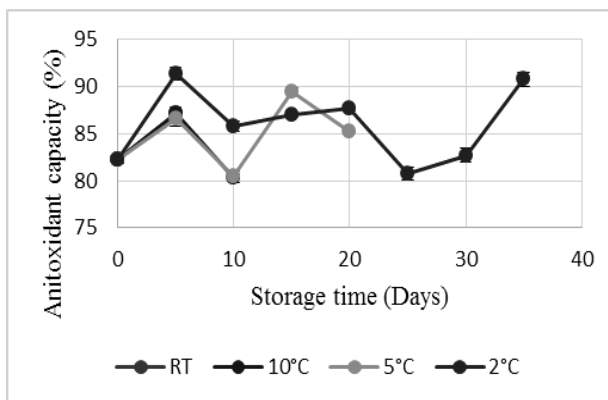


**Figure 8.** Effects of temperatures on L-ascorbic acid of broccoli

It can be seen from Figure 9 that total phenolic contents increased by the storage time at different temperature. TPC of broccoli stored at 5°C and RT was increasing sharply to 77.27 and 75.79 mg GAE/100g, respectively. While 2°C and 10°C raised gradually until the end of storage. Lemoine et al. [22] reported that heated air treated broccoli florets gave higher phenolic compounds. De-compartmentation caused releasing of phenolic compounds. This reason could explain for the increasing amount of phenolic compounds during storage [17].



**Figure 9.** Effects of temperatures on total phenolic compounds of broccoli



**Figure 10.** Effects of temperatures on antioxidant capacity of broccoli

Figure 10 shows antioxidant capacity of broccoli was fluctuated during storage at different temperature. The phenolic compounds and vitamin C are the major antioxidants in broccoli [15]. However, the contribution of vitamin C to antioxidant capacity is minimal comparing to phenolic compounds that are a large group of secondary metabolites in the plant kingdom and their most important biological active is the antioxidant activity [23]. Besides, Fernández-León et al. [9] also found a relationship between chlorophylls content and antioxidant activity. These observation may be causes the fluctuation of antioxidant capacity.



## **4 CONCLUSIONS**

In general, 2<sup>0</sup>C could be suggested as appropriate temperature for storing broccoli for 15 days. After 35 days, although firmness, total phenolic content and antioxidant capacity were significantly higher as compared with the fresh sample, the appearance was dropped to score 1.

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## **EFFECTS OF PH AND SOLVENTS ON THE YIELD AND QUALITY OF PINEAPPLE PEEL PECTIN**

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### **ABSTRACT**

This study was carried out to evaluate impacts of different extraction solvents including HCL, HNO<sub>3</sub> and citric acid and extraction pH on pectin properties obtained from pineapple peels. The obtained results showed that different pH and extraction solvents significantly affected the pectin yields. The highest yield was obtained from using citric acid at pH 1.0, followed by HCL HNO<sub>3</sub> at the same pH. Except the degree of esterification (%), other properties including water holding capacity (%), solubility (%), emulsion activity (%), emulsion stability (%) and galacturonic acid content (%) were insignificantly affected by different acid extraction solvents. Levels of pH were also significantly affected the pectin yield. Increasing of pH led to decreasing on the yield.

**Keywords:** degree of esterification, emulsion properties, extraction solvents, extraction pH, pineapple peel pectin,

### **1 INTRODUCTION**

Pectin is a complex mixture of polysaccharides containing 1, 4-linked  $\alpha$ -D galactosyluronic residues [1]. These blocks of polygalacturonic acid can be esterified to methylesters and free acid groups. Pectin can be commonly extracted from plant tissues; however, the sources that could be used for commercial production are limited. Nowadays, the main sources of pectin extraction are citrus peels or apple pomaces, wastes of juice manufacturing. Ability to form gels is the most important standard to appreciate the uses of pectin, especially in application of jams, candy and gum. In food industry, pectin has been widely used as stabilizer, thickener in beverage, dairy, confections [2]. Pectin also brings many conveniences for the pharmaceutical industry as a transit regularization of gastric contents [3].

Depending on degree of esterification (DE), in the processing industries, pectin is divided into two groups: high methylester pectin (HMP) and low methylester pectin (LMP) [4]. DE values of LMP are from 20 to 40% while 60 to 70% of DE shares of HMP. LMP can be applied in dairy and dietetic products because the gel setting is possible with a low soluble solid content and at high pH value [4]. The block wise carboxyl groups distribution of HMP is provided the suitable condition for pectin stabilization in acidic milk drinks [4].

Water holding capacity was known as the important key to indicate the physical property of pectin [5]. Homogalacturonan is one of pectin structural units which used in food and in other products such as pharmaceuticals, cosmetics, thickening and gelation agents as the hydrocolloids [6]. The functional properties allow pectin to form gel, provide viscosity, stabilize protein and act as a fat mimetic [7]. The development of centrifugation method was used to determine the water holding capacity of lignin, cellulose, pectin, whole locust bean gum and insoluble locust bean gum [8].

The solubility properties of the polymer could be determined by the structural characteristics of pectin and the distribution of hydrophilic and hydrophobic groups on the molecule [9]. Generally, pectin is solubilized in pure water. Pectin powder, when added to water, had a current to hydrate very quickly to form clumps [10]. In industry, formation of clumps can be prevented when mixing pectin powder with water-soluble carrier material or improving pectin's dispersibility through special treatments during manufacturing [11].

Emulsifying properties have included the emulsifying activity (EA) and emulsifying stability (ES) [12]. Pectin has been used in a large scale of industry which is not only in fruit converse, beverage and dairy products [5] but also in meat, ice cream and baked products [12]. EA has showed the solubilization or dispersion of two liquids that mixed together while ES determined the stability of an emulsion [13].

It has been reported that pH levels and solvents showed significant roles in pectin extraction [14]. According to a study of Khan *et al.* [15], different pH levels ranging from 1-5 resulted in the yields from 1.0-3.0. Using hydrochloric acid [16] nitric acid [17] and citric acid [18] [19] [20] [21] for extracting pectin from plant materials have attracted increasing attention. Among those, citric acid was the potential solvent for the extraction pectin [21] [22]. Therefore, extraction solvents used in this study were hydrochloric acid, nitric acid and citric acid at pH 1, 2, 3.

Pineapple is an important tropical fruit which is the second harvest after bananas, contributing over 20 percent of the production on world (FAO). Nearly 70% of fresh fruit is consumed by producing countries. 50% of total output is from the producers of Thailand, Philippines, Brazil and China. Pineapple (*Ananas comosus*) is one of the main agricultural commodities of Indonesia which 135, 000 tons were produced in the year of 2014 [23]. In the year of 2017, the production of pineapple of Tan Phuoc ward, Viet Nam was 201, 000 tons (tiengiang.gov.vn); followed by Kien Giang and Long An. Exportation of canned pineapple was taken advantage with the increasing 0.3% per year (agro.gov.vn). In 2011, the first factory produced pineapple juice with yield of 2 million liters per year. Many cultivars of pineapple are grown, however *Cayena lisa* is the most spread variety. The composition contents of this cultivar include pulp (33%), core (6%), peel (41%) and crown (20%). The fruit is containing many minerals such as calcium, sodium, phosphorus with high moisture content (81.2 to 86.2%). It is a rich source of vitamin C and usually consumed as juice or dessert. Moreover, pineapples are processed to various products

including canned fruit, dried pineapple, juice, nectar, jelly, jams, candy ect. contributing a large consumption not only in Vietnam but also over the world. The huge waste from processing, which urgently to be overcome, could be the issue for the manufacturer and also the environment. Pectin extraction using pineapple peels should be considered as one of potential solutions for the problem.

There have been many researches focusing on extracting pectin from the common sources such as citrus peel, apple pomace however the study on pineapple peel still is currently lack of. Therefore, this study was carried out to determine influences of pH and solvent extraction on the quality of crude pectin extracted from pineapple peels.

## **2 MATERIALS AND METHODS**

### **2.1 Sample preparation**

#### *2.1.1 Materials*

The sound fruits were collected from a farm at Long An province, Vietnam with the identical green color for all samples. The fruits were washed under the tap to remove debris, insects before being peeled and dried using an air-oven (WiseVen, Wisd Laboratory Instruments, Korea) at 65°C until constant weight. The dried peels then were milled into a powder form using a grinder (Philips HR2115, Indonesia). The peel pineapple powder were packed in polyethylene bags and stored in desiccator for further extraction. All experiments were done in three replications.

#### *2.1.2 Chemicals*

All chemicals using in this research included hydrochloric acid (Merk, German), nitric acid (Merk, German), citric acid, sodium chloride (Merk, German), polygalacturonic acid with 97% purity (Sigma-Aldrich, USA), pectin standard with 65% MED (Himedia, India), 3,5-dimethyl phenol reagent, monohydrated D-galacturonic acid, concentrated sulphuric acid, absolute ethanol, sodium benzoate and commercial soybean oil were purchased from a local supplier. Nanopure water was prepared from double distilled water using Barnstead EasyPure II machine (Thermo Scientific, USA).

### **2.2 Analytical methods**

#### *2.2.1 Extraction of pectin from pineapple peels*

The procedure was mainly based on a reported research of Nguyen and Savage [24] with minor modifications. The dried peel powder was mixed with aqueous solution of hydrochloric acid, nitric acid, citric acid at pH 1.0, 2.0, 3.0. The mixture was then heated to 80°C in 20 minutes and cooled down to room temperature before being filtered through four layers of cheesecloth. The filtrate was precipitated by mixing with the double volume of 96% ethanol and stirred slowly for 15 min and stored at 4°C for 24 hours. The precipitated pectin was collected by centrifugation at

3500 rpm for 15 minutes then washed with 70% ethanol and then 96 % ethanol to remove more soluble alcohol impurities. The pectin pellets were dried in a freeze-dryer (Labcono FreeZone, US) and then ground to fine powder, stored in dark glass bottle until further analysis commenced.

The yield of pectin was calculated using dry basis equation

$$\text{Yield\%} = \frac{\text{Pectin weight}}{\text{Sample weight}} \times 100$$

Where in: sample weight is the initial amount of pineapple peel powder and pectin weight is the weight of dried pectin powder obtained.

### 2.2.2 Measurements of Degree of esterification using FTIR

The degree of esterification (DE) was determined by Fourier- Transform Infrared Spectroscopy (FTIR) following Singthong *et al.*, [25]. In details, 0.5g of extracted pectin powder was mixed with KBr at ratio of 90:10 (KBr: pectin powder) before measuring FTIR using a FTIR Spectrometer (Tensor 27, Bruker, Germany). If the DE value was higher than 50%, pectin could be categorized as high methoxyl pectin (HMP) and low methoxyl pectin (LMP) would have DE lower than 50% [26]. The absorbance of the asymmetrical stretching vibration carboxyl groups (1600-1630 cm<sup>-1</sup> wavelength number) and the carbonyl groups originated from carbonyl and carbomethoxyl groups (1730 – 1760 cm<sup>-1</sup> wavelength number) were measured for the calculation of DE following this equation with three replications.

$$\text{DE(\%)} = \frac{A_{(1730-1760)}}{A_{(1600-1630)} + A_{(1730-1760)}} \times 100$$

Where:

$A_{(1600-1630)}$ : Absorbance intensity of the free carboxylate groups

$A_{(1730-1760)}$ : Absorbance intensity of the esterified carboxylic groups

$A_{(1600-1630)} + A_{(1730-1760)}$ : Absorbance intensity of total number carboxylic groups

### 2.2.2 Determination of water holding capacity and solubility of extracted pectin

Centrifugation technique was developed to determine solubility and water holding capacity (WHC) by modifying methods of Eastwood *et al.*, [27] and Al-Sheraji *et al.*, [5]. Briefly, 0.3-0.5g of pectin was soaked in 20ml of Nanopure water and left to stand for 1 hour at room temperature. After that, the supernatant was removed by centrifugation at 6000rpm for 15 minutes. Then, the pellets were left to drain for 30 minutes. The pellets were dried in oven until the constant weight was obtained. The solubility was calculated by the equation:

$$\text{Solubility} = m_i - m_d (\%)$$

$m_i$ : initial weight of dried pectin samples before WHC processing

$m_d$ : dried weight of pectin samples after WHC processing

Water holding capacity was measured as same as the method of solubility. The changes in weight of pellet before and after drying were recorded for the calculation of WHC:

$$WHC = \frac{m_w - m_d}{m_d}$$

Where:

$m_w$ : wet weight of pectin samples

$m_d$ : dried weight of pectin samples after WHC processing

### 2.2.3 Determination of emulsifying properties of extracted pectin

The pectin emulsifying activity was determined by a research of Sciarini *et al.*, [12] and Baississe *et al.*, [28] and based on the ratio of emulsified layer volume and the whole volume of solution. In a test tube, 8ml of pectin solution (0.5% w/v) was added with 8ml of soybean oil. The mixture was homogenized in a vortex mixer at the high speed for few minutes at RT. After centrifugation at 800g in 10 minutes [12], the emulsified layer volume was separated for determination as  $E_{LV}$  and the whole volume of solution was  $W_v$ . The calculated quation for emulsifying activity was followed as:

$$EA = \frac{E_{LV}}{W_v} \times 100 \quad [28]$$

### 2.2.4 Determination of galacturonic acid (GalA) content of extracted pectin

The GalA content was determined using the 3, 5-dimethyl phenol reagent, monohydrated D-galacturonic acid (Sigma, USA) was used as standard [29]. Samples which contained 0.125ml of pectin solution (400 $\mu$ g/ml) and 0.125ml of 2% NaCl were treated with 2ml concentrated sulphuric acid, then be mixed. The mixture was heated to 70°C for 10 minutes and cooled at RT. Finally, 0.1 ml of 3, 5-dimethyl phenol reagent (0.1g 3, 5-dimethyl phenol in 100ml glacial acetic acid) was added. A yellow color developed. After that, the absorbance was measured at 450 nm and 400nm using an UV Spectrophotometer with a standard curve with GalA at 200, 400, 600, 800 and 1000  $\mu$ g mL<sup>-1</sup>.

The difference of absorbance at 450 nm and at 400nm was used to calculate the concentration of galacturonic acid per 1 mL. GalA% was calculated by the equation

$$GalA\% = \frac{GalA \text{ concentration } \mu\text{g/ml}}{400 \mu\text{g/ml}} \times 100\%$$

Where:

GalA concentration ( $\mu$ g/ml) was determined by standard curve construction.



### 2.2.5 Statistical analysis

In each treatment, samples were done in triplicate. Datas and results were then presented as the mean of three determination  $\pm$  SD. Statistical analysis was performed using SPSS software version 20 with 95% level of confidence to assess the effects of different types of acidic solvents and enzymatic treatments on the yield and properties of pectin obtained from pineapple peel.

## 3 RESULTS AND DISCUSSION

### 3.1 Effects of pH and extraction solvents on the yield of pineapple peel pectin

Table 1 showed that at the same pH, there were insignificantly different effects between extraction solvents on the pectin yield. This is in agreement with the results obtained from studies of Virk and Sogi [30] and Canteri-Schemin *et al.* [31] in which the yields of pectin extracted from apple using hydrochloric acid, nitric acid and citric acid were compared. Between the two inorganic acids, it was observed that there was no significant difference in the yield, in spite of the effects of nitric acid being slightly larger than hydrochloric acid, and a low pH was necessary to improve the yield (Table 1). The yield of pectin extraction using HCL and HNO<sub>3</sub> ( $0.4 \pm 0.0$  and  $0.8 \pm 0.0\%$ , respectively) from pineapple peels observed from Ukiwe and Alinnor [32] were much lower than this study. Using different acid types strongly influence the macromolecular and gelling properties of isolated pectin and the citric acid being the least pectin degrading extracting agent [33]. The strong acid solution might lead to smaller pectin particles due to partial hydrolysis [24].

The less amount of pectin isolated might be due to some pectin were attached to the cell wall components although pectin molecules can be partially solubilized from plant tissues without degradation by weak acidic aqueous solvents [34]. Joye *et al.* [35] found that strong acidic condition (pH < 2.0) would extract the non-calcium sensitive pectin and the remaining primarily calcium sensitive-pectin in citrus peel.

At the same extraction solvents, pH ranging from 1.0-3.0 resulted in significant differences on the pectin yield. It has been seen from Table 1 that higher pH of extraction solvents resulted less in pectin yield. The extraction parameters were maintained at 20 minutes and 80°C. Kertesz [36] reported that the more hydrogen ions concentration present in the solvent or at low pH stimulates the hydrolysis of protopectin meanwhile the maximum yield of pectin was obtained at extraction solvent pH 1.



**Table 1:** The yield and pectin properties extracted by different solvents at different pH

pH	Solvents	Yield (%)	WHC	Solubility (%)	DE (%)	GalA (%)	EA (%)
1	HCL	2.0 ± 0.0 <sup>a,A</sup>	5.5 ± 0.6 <sup>a,A</sup>	27.8 ± 3.7 <sup>a,A</sup>	49.1 ± 1.4 <sup>b,A</sup>	58.1 ± 9.7 <sup>a,A</sup>	54.0 ± 3.6 <sup>a,A</sup>
	HNO <sub>3</sub>	2.2 ± 0.1 <sup>a,A</sup>	6.5 ± 1.9 <sup>a,A</sup>	30.6 ± 4.9 <sup>a,B</sup>	56.7 ± 3.2 <sup>ab,A</sup>	58.9 ± 2.9 <sup>a,A</sup>	49.6 ± 0.7 <sup>a,A</sup>
	Citric acid	2.3 ± 0.3 <sup>a,A</sup>	7.2 ± 2.4 <sup>a,A</sup>	29.7 ± 4.1 <sup>a,A</sup>	58.1 ± 2.5 <sup>a,A</sup>	39.3 ± 6.4 <sup>b,B</sup>	50.8 ± 1.4 <sup>a,A</sup>
2	HCL	1.4 ± 0.1 <sup>a,B</sup>	5.1 ± 0.9 <sup>a,A</sup>	36.0 ± 5.5 <sup>a,A</sup>	43.1 ± 2.4 <sup>b,B</sup>	56.6 ± 4.0 <sup>ab,A</sup>	58.5 ± 5.5 <sup>a,A</sup>
	HNO <sub>3</sub>	1.4 ± 0.2 <sup>a,B</sup>	5.2 ± 0.4 <sup>a,A</sup>	34.0 ± 1.5 <sup>a,B</sup>	44.5 ± 1.8 <sup>ab,B</sup>	59.8 ± 2.5 <sup>a,A</sup>	49.1 ± 3.8 <sup>b,A</sup>
	Citric acid	1.6 ± 0.0 <sup>a,B</sup>	4.2 ± 0.3 <sup>a,B</sup>	31.0 ± 4.8 <sup>a,A</sup>	48.4 ± 1.8 <sup>a,B</sup>	55.2 ± 5.5 <sup>b,AB</sup>	53.6 ± 1.5 <sup>ab,A</sup>
3	HCL	1.1 ± 0.0 <sup>a,C</sup>	5.6 ± 1.4 <sup>a,A</sup>	34.6 ± 1.6 <sup>ab,A</sup>	44.5 ± 1.5 <sup>b,B</sup>	52.1 ± 6.5 <sup>a,A</sup>	54.0 ± 3.6 <sup>a,A</sup>
	HNO <sub>3</sub>	1.3 ± 0.1 <sup>a,B</sup>	5.9 ± 0.7 <sup>a,A</sup>	43.4 ± 1.8 <sup>a,A</sup>	42.1 ± 1.5 <sup>b,B</sup>	56.9 ± 1.9 <sup>a,A</sup>	51.1 ± 5.5 <sup>a,A</sup>
	Citric acid	1.3 ± 0.0 <sup>a,C</sup>	5.5 ± 0.3 <sup>a,C</sup>	19.5 ± 1.9 <sup>b,AB</sup>	55.1 ± 2.9 <sup>a,A</sup>	58.0 ± 7.1 <sup>ab,A</sup>	50.8 ± 1.5 <sup>a,AB</sup>

The same letters <sup>a-c</sup> presented in the same pH, the same letter <sup>A-C</sup> presented in the same solvent indicate non-significant difference ( $p < 0.05$ )

### 3.2 Effects of pH and extraction solvents on water holding capacity

Table 1 showed that there were not significant differences of solvent effects on WHC. The WHC values were ranging from  $4.2 \pm 0.3$  to  $7.2 \pm 2.4$  (g H<sub>2</sub>O /g dry sample) (Table 1). After centrifugation, there was the existence of residues not being retained in the bottom of falcons; therefore they were taken along with the water. The obtained results were much lower than pectin extracted from citrus fruit ( $37.9 \pm 2.0$  g/g dry sample) [8]; however comparable to those extracted from a study of Al-Sheraji *et al.* [5]  $8.8 \pm 0.2$ g/g sample for *Mangifera indica* L. fiber and  $12.4 \pm 0.4$  g/g sample for cellulose.

Water holding capacity has been proven as one of the most important physical property of pectin [5]. It was determined by the amount of water held by the fibrous matrix that benefited on the human gut [37], upper and lower intestine [38]. Last but not least, in the upper intestine, WHC might have the effects on the nutritional absorption process, postprandial safety and the motility of the intestinal tract [38]. More specifically, the constitutions, especially pectin and the guar might form a gel which could enhance the speed of glucose absorption [39]. In food, WHC plays a major role in the formation of texture and the influence on the quality characteristic of finished food product as the additives.

### 3.3 Effects of pH and extraction solvents on solubility

In this present study, the solubility of pectin extracted using acidic solvents at different pH levels was determined. At pH 1.0 and 2.0, there were no significant differences on the solubility of pectin obtained from HCL, HNO<sub>3</sub> and citric acid. However the significant difference of different solvents was obtained at pH 3.0. Citric acid samples had the least percent of solubility excepting those at pH 1.0.

In general, solubility of pectin in water is increased by minimizing the polymer size and increasing the methoxy–ester groups; however, the pH, temperature, and the solutes concentration in the environment should be importantly considered [40] [41]. It was clearly seen that the solubility was higher when the pH of hydrochloric acid and nitric acid increased. It could be explained that the solubility of extracted pectin using hydrochloric acid could be reduced due to the oxidation of plant tissue constituents and drying process [42]. Citric acid was an organic acid, therefore owning weaker oxidation capacity as compared to the two strong inorganic acids.

### **3.4 Effects of pH and extraction solvents on the degree of esterification of pineapple peel pectin**

The existing of absorptions at around 1600 and 1750  $\text{cm}^{-1}$  were from free and esterified carboxyl groups, respectively. The degree of esterification (DE) of extracted pectin ranged from  $42.1 \pm 1.5$  to  $58.1 \pm 2.5\%$  (Table 1). The pectin samples extracted using  $\text{HNO}_3$  and HCL at pH 1.0 and citric acid at pH 3.0 in this study can be categorized as high methoxyl pectin (HMP) with DE values ranging from  $55.1 \pm 2.9$  to  $58.1 \pm 2.5\%$  (Table 1) therefore they could be considered as the slow set pectins.

It could be seen that almost pectin samples extracted from pineapple peel showed low DE values (Table 1). There was a slight decrease when pH increased from 1.0 to 2.0. At different pHs, there were significant differences on %DE of pectin obtained from same extraction solvent.

### **3.5 Effects of pH and extraction solvents on galacturonic acid content of pineapple peel pectin**

Galacturonic acid ranged from  $39.3 \pm 6.4$  to  $59.8 \pm 2.5\%$  when the peel extracted by different acid (Table 1). The highest GalA content was  $58.9 \pm 2.5\%$  at pH 1.0 using nitric acid. The results from this current study indicated that extraction solvents significantly affected GalA content of pectin obtained at the same pH. With GalA > 50%, it was indicated that pectin extracted from pineapple peels using inorganic acids (HCL and  $\text{HNO}_3$ ) at pH 1.0 and 2.0 were more purify than those using organic acid (citric acid). There was no significant different between the different levels of pH on %GalA, except in citric acid. It was an increase of %GalA when using citric acid at pH increasing ranged from  $39.3 \pm 6.4$  to  $58.0 \pm 7.1\%$ . The low %GalA content of pectin extracted using citric acid at pH 1-3 indicated that pectin was mixed with other constituents.

The purity of pectin or galacturonic acid content would be affected by pH [14]. In this study, the %GalA increased with the decrease of pH of HCL (Table 1). In contrast, %GalA increased with the increase of pH of nitric acid and citric acid solvents. This observation was similar to obtained results of a study of Sotanaphun *et al.* [14] in which increasing of pH could lead to the higher %GalA. On the other hand, the increase in galacturonic acid was probably due to the loss of ash after extraction [36].

### 3.6 Effects of pH and extraction solvents on emulsifying properties of pineapple peel pectin

#### *Emulsion activity*

The emulsion activity of pectin samples were performed by the investigation of oil-in-water emulsions which containing 0.5% (w/w) pectin solutions. After process of centrifugation, three phases of emulsions were observed in the order of the oil tiny layer on the top, pectin dispersed into aqueous phase at the bottom and finally the emulsified layer phase existed in the middle. The differences in emulsion properties of pectin might be based on the existence of different protein contents in pineapple peel and neutral sugar side chain in pectin structure [42].

The current results indicated that the samples extracted by hydrochloric acid providing the highest EC values,  $54.0 \pm 3.6\%$ ;  $58.5 \pm 5.5\%$ ;  $54.0 \pm 3.6\%$  at pH1, 2, and 3, respectively while the other acids had lower values.

At pH 1.0 and 3.0, there were insignificant differences on EA value between pectin samples extracted using different solvents at the same pH. However, at pH 2.0, the highest EA value obtained from using HCL ( $58.5 \pm 5.5\%$ ).

#### *Emulsion stability*

The stability of pectin samples were observed within 30 days at 4°C. The treatment was opaque and creamy with the homogenized solution. On the first day, it was stable for all emulsions. After 7 days, a cream layer started to appear for all emulsions stabilized by 0.3% pectin. On the 14<sup>th</sup>, the color of solution became more and more transparent with the appearance of residues on the bottom of the tubes. The following days would showed as same as the 14<sup>th</sup> day until the absolute change in the 28<sup>th</sup> day. At the end of a 30-day storage period, a transparent layer at the bottom was observed for all emulsion prepared. It was similar to the observation of Kermani *et al.*, [29].

## 4 CONCLUSIONS

The results from this study showed the effects of extraction solvents on the quality of pectin extracted from Vietnamese pineapple peel. Different pH levels were significantly impacted on the yield of pectin extraction; meanwhile the types of extraction solvents were not. Generally, almost pectin extracted from the current work could be categorized as low methoxyl pectin ( $DE < 50\%$ ) however, pectin extracted using  $HNO_3$ , HCL at pH1 and citric acid at pH3 could be categorized as high methoxyl pectin ( $DE > 50\%$ ). With high methoxyl content, pectin samples have been more desirable and diversity application in food industry meanwhile LMP were appropriate for low fat/reduced calories foods. Compared to other sources, pineapple peels contained less pectin; however, the isolated pectin has possessed the potential gelling, emulsifying and stabilizing capacities.

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## **EFFECTS OF PHYSICOCHEMICAL TREATMENT ON DRAGON FRUIT (*HYLOCEREUS POLYRHIZUS*) QUALITY BEFORE STORAGE.**

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### **ABSTRACT**

This study was conducted to determine the effect of hot water treatment temperature in combination with pH adjustment of 3.5 on the quality of dragon fruits before storage. The dragon fruits were treated with hot water, adjusting pH 3.5 at different temperature ranges from 46 to 52°C in the periods from 6 to 15 minutes. The results showed that hot water treatment temperature and time combined with pH adjustment of 3.5 affected significantly ( $p < 0.05$ ) on physiological and biochemical characteristics of dragon fruits. The results indicated that the treatments at high temperatures for a prolonged period significantly ( $p < 0.05$ ) affected the quality leading to darker red color and increased weight loss as well as reduced firmness, Vitamin C content and total acidity of the dragon fruits. The dragon fruits treated with hot water at 50°C for 12 minutes and adjusted pH to 3.5 showed higher quality than those treated in other treatments. The shelf-life and quality of the dragon fruits can be prolonged and maintained, respectively by hot water treatment combined with immersion of fruits in pH 3.5.

**Keywords:** Dragon fruit, Hot water treatment, pH adjustment, Shelf-life

### **1 INTRODUCTION**

Dragon fruit (*Hylocereus polyrhizus*) has an attractive color, shape and rich source of fiber, vitamin C and minerals [9]. It also has phytoalbumins which are highly valuable for their antioxidant properties. Due to having less sugar content than most popular tropical fruits, dragon fruits are more suitable for the patients who suffer from diabetics and high blood pressure. These attributes have led people to consider it as a healthy and premium fruit [6]. Locally, dragon fruits are sold mainly for fresh consumption. Physical, biochemical and physiological deterioration as well as damages caused by disease and spoilage affect quality and increase postharvest losses of fruits after few days of storage under ambient conditions. There is a growing demand for dragon fruits in nearby countries such as China, Hong Kong and Singapore. Dragon fruits show good



potential to be exported to Japan, Korea, USA, Australia, New Zealand and European countries, due to their unique flavors and tastes.

Hot water treatments of fruit after harvest have been demonstrated to protect horticultural produces against postharvest decay [2, 3, 5, 8, 13, 14]. Hot water treatments directly or indirectly inhibit the pathogens present on horticultural produces [7]. Heat treatment technologies are currently relatively simple, non-chemical alternative to methyl bromide that can kill quarantine pests in perishable commodities, as well as control some postharvest diseases. Unlike methyl bromide, heat treatments do not pose significant health risks from chemical residues, as a result, are more appealing to consumers than methyl bromide fumigation [4]. However, long-term heat treatment can affect the sensory quality (color, texture, taste), nutritional value (minerals, vitamins, especially vitamin C) and other physicochemical properties (firmness, respiration, acidity, sugars and total soluble solids) of the dragon fruits. The hot water treatment of fruits often results in loss of solute due to heat acting to break up the cell and the substances dissolve in water and escape from the fruits. It is suggested that the use of essential chemicals helps maintain fruit quality. Citric acid is capable of inhibiting polyphenoloxidase activity and anti-browning activity, which can maintain the color of the fruit during storage. In addition, fruits treated with citric acid limit sugar and acid damage, thus maintaining their quality. Treatments with citric acid to adjust the solution to the desired pH and non-toxic help minimize microorganisms in the fruits. Acids have the ability to limit enzyme activity and prevent or delay browning which can maintain the color of the fruit during storage. Zauberman et al [15] studied the method of treating fruits in low pH solution to improve peel color by inhibiting polyphenol oxidase enzyme leading to browning and maintaining fruit quality

This study focused on postharvest handling of dragon fruits using hot water treatment in combination with pH 3.5 to extend shelf-life, reduce the damage and produce better quality of dragon fruits for domestic markets and exports. The aim of this study, therefore, was to evaluate effects of physicochemical treatment on dragon fruit (*Hylocereus polyrhizus*) quality before storage.

## **2 MATERIALS AND METHODS**

### **2.1 Preparation of dragon fruit materials**

The dragon fruits (*Hylocereus undatus*) harvested in Tien Giang and Long An provinces were provided by Hoang Phat Co. Ltd. The dragon fruits selected were the white flesh cultivar weighing about 350-500 grams.

### **2.2 Hot water treatment**

The dragon fruits were soaked in a water tank that controls temperature to monitor the treatment temperature and time. The treatment temperatures tested in this study were 46°C, 48°C, 50°C, 52°C, and the treatment times were 6, 9, 12 and 15 minutes.

## 2.3 Chemical treatment

Citric acid was used to adjust the pH of the treatment water to 3.5.

## 2.4 Quality assessments

- Dragon fruit colors were measured by using KONICA MINOLTA CR410 color meter.
- Weight losses (%) were determined by the technical balance
- Total soluble solids (TSS) were measured by an ATAGO RX-5000 refractometer.
- Total acid contents were determined by titration with 0.1 N NaOH using Titroline SCHOTT Instruments.
- Vitamin C contents were determined by titration of 2,6 dichloro indophenol phenolate sodium (2,6-DCPIP) using Titroline SCHOTT Instruments.
- Total numbers of mold and yeast spores were counted on the 3M™ Petrifilm™ Yeast and Mould Count Plate.
- The evaluation of fruit sensory quality was performed by establishing the sensory board and the sensory criteria which scores for four main indicators: color, smell, taste, and appearance of the dragon fruits. The sensory board was trained in the term and termination corresponding to the agreed quality descriptions and scores.

## 2.5 Data analysis

Data were collected and processed using ANOVA and correlations between factors affecting fruit quality characteristics were identified using JMP 9.0, MINITAB 16 and Excel software.

# 3 RESULTS AND DISCUSSION

## 3.1 Physicochemical properties of fresh dragon fruits

**Table 1:** Physicochemical properties of fresh dragon fruits

Parameters	Unit	Values
Color	L*	65.25 ± 0.11
	a*	2.47±0.04
	b*	-0.22 ±0.06
Peel firmness	Kg/cm <sup>2</sup>	6.8±0.34
Flesh firmness	Kg/cm <sup>2</sup>	1.04 ±0.07
Weight	gram	362.15 ± 5.11
Respiration rate	mg/kg/h	42.25± 3.11

Parameters	Unit	Values
Total soluble solids	°Brix	11.89 ± 0.24
Total acidity	mg/100g	7.01± 0.15
Total sugar content	%	8.53 ± 0.17
Vitamin C	mg/100g	10.72± 0.24

### 3.2 Effects of hot water treatment temperature and time combined with pH of 3.5 on the respiration rate (mg CO<sub>2</sub>/kg/h) of dragon fruits

**Table 2:** Respiration rate (mg CO<sub>2</sub>/kg/h) of dragon fruits after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	39.39 ±0.19	39.32±1.19	39.55 ±1.35	38.38±1.25
48	38.26±0.93	3864± 0.63	38.63 ±1.47	38.56±1.41
50	38.48±1.22	38.31±2.57	37.78±2.47	37.51± 0.87
52	37.48 ±1.28	36.62 ± 2.51	34.05±1.72	34.43 ± 1.12

The respiratory rate of the dragon fruits after hot water treatment with low pH (pH 3.5) decreased compared with the control samples. The respiration rate of fruits tended to slightly decrease as the treatment temperature and time increased since heat slows the fruit ripening process by reducing the activity of Aminocyclopropane-1-carboxylic acid (ACC) oxidase which is an enzyme to synthesize ethylene.

### 3.3 Effects of hot water treatment temperature and time combined with pH of 3.5 on the weight loss of dragon fruits

**Table 3:** Weight loss (%) of the fruit after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	0.20 ± 0.66	0.04±0.53	-0.28±0.15	-0.56±0.30
48	0.24±0.42	0.58±0.42	-0.18±0.44	-0.44±0.29
50	0.80±0.39	0.58±0.37	-0.16±0.32	-1.42±0.23
52	1.16±0.71	0.98±0.66	-0.96±0.35	-3.81±0.23

After harvesting, the transpiration of fruits increases since fruits no longer get water supplied from the roots as in the trees [10]. The weight loss increased as the hot water temperature and time increased ( $p < 0.05$ ). However, long treatment time causes water penetration into fruit cells, increasing fruit weight and reducing shrinkage due to the interaction between treatment temperature and time. The interaction between temperature and time of the treatment also affected the weight loss of dragon fruits. Table 3 showed that the weight of dragon fruits treated at 50-52°C for 15 minutes increased due to water penetration, whilst high temperature treatment in short time (6 minutes) reduced the weight. This can be explained that high temperatures cause high weight loss [12]. In addition, there was no significant difference in weight loss among treated dragon fruits.

### 3.4 Effects of treatment temperature and time with hot water combined with pH of 3.5 on the firmness ( $\text{kg}/\text{cm}^2$ ) of dragon fruits

**Table 4:** Firmness ( $\text{kg}/\text{cm}^2$ ) of dragon fruit peel after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	4.70 ± 0.66	4.64±0.56	6.08±0.11	4.46±0.30
48	4.94±0.72	4.58±0.52	4.98±0.44	5.24±0.29
50	4.00±0.27	3.58±0.37	4.06±0.32	2.92±0.23
52	4.56±0.74	4.98±0.66	4.56±0.35	2.88±0.23

The firmness of dragon fruits treated with hot water (pH = 3.5) was significantly ( $p < 0.05$ ) lower than that of the control samples ( $6.8 \pm 0.34 \text{ kg}/\text{cm}^2$ ). However, there was no significant difference between the treatments, except the treatment at 50-52°C for 15 minutes, caused softening of the fruits. In addition, there was no significant difference in fruit firmness between control flesh samples ( $1.14 \pm 0.15$ ) and treated flesh samples at 46 - 52° C for 6 to 15 minutes (Table 4).

**Table 5:** Firmness ( $\text{kg}/\text{cm}^2$ ) of dragon fruit flesh after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	1.10 ± 0.61	1.14±0.56	1.08±0.11	1.16±0.31
48	1.14±0.32	1.18±0.22	0.98±0.41	0.94±0.29
50	1.00±0.27	1.08±0.37	1.06±0.12	1.02±0.41
52	0.93±0.57	0.98±0.68	0.86±0.47	0.88±0.32

### 3.5 Effects of hot water treatment temperature and time combined with pH of 3.5 on the total soluble solids of dragon fruits

**Table 6:** Total soluble solids of dragon fruit after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	11.36 ± 0.20	11.14 ± 0.18	10.96 ± 0.24	10.85 ± 0.37
48	11.42 ± 0.14	11.28 ± 0.16	11.23 ± 0.19	11.26 ± 0.14
50	11.27 ± 0.22	11.35 ± 0.21	11.24 ± 0.22	10.88 ± 0.09
52	11.31 ± 0.14	11.22 ± 0.31	11.05 ± 0.21	10.36 ± 0.24

The TSS of the treated samples decreased significantly ( $p < 0.05$ ) compared with the control sample ( $11.89 \pm 0.24$  °brix). However, there was no significant difference between the TSS of the samples treated at different temperatures and times, except that the samples at 50-52°C for 15 minutes had lower TSS than other dragon fruit samples. The reduction of TSS in dragon fruit was due to the lack of starch transformation into sugars [1, 11].

### 3.6 Effects of hot water treatment temperature and time combined with pH of 3.5 on the total acidity (mg/100g) of the dragon fruits

The main acid in the fruit is citric acid, which is produced during ripening. The results showed that the acid content decreased when treated at high temperature and in long time. Especially, the dragon fruits treated at 50°C for 15 minutes and at 52°C for 12-15 minutes showed higher level of acidity than the control samples and the other treated samples. However, the level of total acidity reduction in hot water treatment samples combined with low pH adjustment (citric acid supplementation) was not so great. The conversion of starch into sugar can also reduce the acid titration in the fruit. The acidity decreases as the temperature increases respiratory rates. The respiration rate of dragon fruits will increase with increased temperature that can cause a decrease in acidity. It is noted that organic acids can be used as a substrate for respiration [13].

**Table 7:** Total acidity content (mg/100g) of the dragon fruit after treatment with hot water combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	6.31 ± 0.33	6.11 ± 0.51	6.76 ± 0.34	6.80 ± 0.18
48	6.78 ± 0.29	6.10 ± 1.93	6.78 ± 0.26	5.50 ± 0.22
50	5.38 ± 0.86	5.97 ± 0.36	5.95 ± 0.19	5.33 ± 0.24
52	6.19 ± 0.46	6.19 ± 0.46	5.48 ± 0.24	5.57 ± 0.10

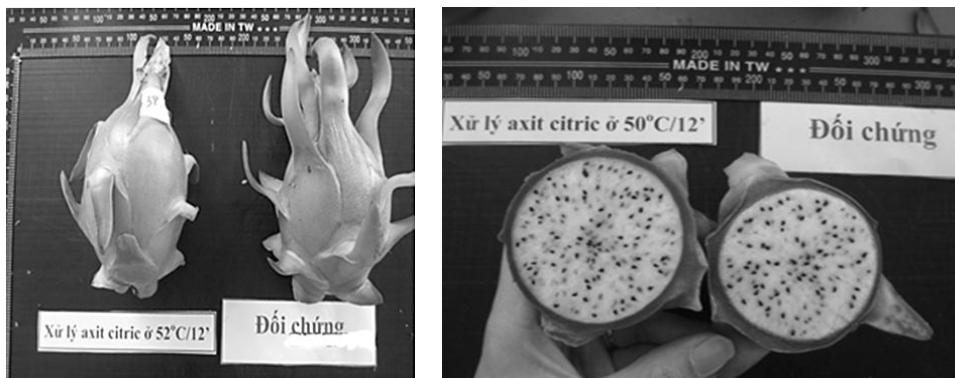
### 3.7 Effects of treatment temperature and time with hot water on vitamin C content (mg/100g) of the treated dragon fruits

**Table 8:** The content of vitamin C (mg/100g) of the dragon fruit after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	7.31 ± 0.33	7.119 ± 0.51	8.12 ± 0.26	6.95 ± 0.60
48	6.78 ± 0.29	10.1 ± 1.93	8.31 ± 0.33	7.18 ± 0.31
50	7.38 ± 0.86	6.97 ± 0.36	7.85 ± 0.28	6.52 ± 0.45
52	6.19 ± 0.46	5.198 ± 0.36	3.84 ± 0.15	3.61 ± 0.25

Vitamin C content of hot water samples treated with hot water at high temperature for a long time combined with a significant decrease in pH ( $p < 0.05$ ). The samples of dragon fruit treated at 52°C for 12-15 minutes had very low vitamin C content. However, there was no significant difference between the treatments at 46, 48°C during the experimental periods and 50°C for 6 to 12 minutes. Therefore, the hot water treatment of dragon fruits at 50°C for 12 minutes combined with pH of 3.5 maintained the vitamin C content relatively high compared to other treatments.

### 3.8 Effects of hot water treatment temperature and time combined with pH of 3.5 on the color (a\* value) of the treated dragon fruits



**Figure 1:** Checking dragon fruit color after hot water treatment combined with pH 3.5

**Table 9:** Color (a\* value) of the dragon fruits after hot water treatment combined with pH 3.5 (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	3.32 ± 0.11	3.14 ± 0.09	3.22 ± 0.07	3.21 ± 0.15
48	3.67 ± 0.15	3.29 ± 0.13	3.61 ± 0.09	3.48 ± 0.11
50	3.35 ± 0.05	4.16 ± 0.09	4.37 ± 0.08	4.41 ± 0.15
52	4.24 ± 0.08	4.32 ± 0.08	4.48 ± 0.05	4.39 ± 0.11

The treated dragon fruits were darker and more red than the control samples (2,47±0,04). The dragon fruits treated at 50°C for 9 minutes or longer and those treated at 52°C for 6 to 15 minutes showed more red than the other samples.

### 3.9 Effects of hot water treatment temperature and time combined with pH of 3.5 on the sensory quality of the treated dragon fruits

**Table 10:** Sensory quality of dragon fruits after hot water treatment combined with pH 3.5 (n=9)

Temp (°C)	Time (min)			
	6	9	12	15
46	19.89 ± 0.11	19.67 ± 0.24	19.44 ± 0.34	19.44 ± 0.34
48	19.22 ± 0.28	19.22 ± 0.32	19.89 ± 0.11	4.67 ± 0.17
50	19.56 ± 0.24	19.22 ± 0.28	19.56 ± 0.24	18.44 ± 0.34
52	18.11 ± 0.11	17.11 ± 0.11	16.56 ± 0.18	15.44 ± 0.18

The sensory quality of dragon fruits was influenced by hot water treatment temperature and time. The treatments at 52°C for 12-15 minutes caused signs of softness, water soaked near the skin and dark red color of the skin.

### 3.10 Effects of hot water treatment temperature and time combined with pH of 3.5 on the inhibition of molds and yeasts growing on the dragon fruits

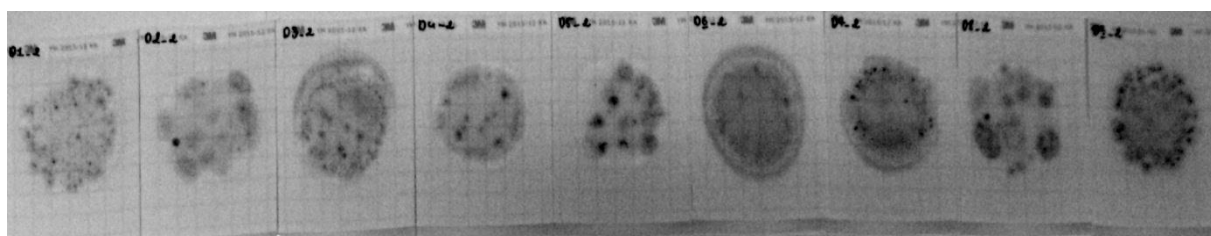
**Table 11:** Effects of hot water treatment temperature and time on the inhibition of molds and yeasts (cfu/cm<sup>2</sup>) growing on the dragon fruits (n = 9)

Temp (°C)	Time (min)			
	6	9	12	15
46	23.83 ± 15.49	32.64 ± 21.05	7.80 ± 5.27	21.23 ± 13.42
48	41.92 ± 20.98	74.74 ± 22.58	80.88 ± 42.09	32.35 ± 15.34
50	234.70 ± 114.91	203.65 ± 83.18	14.44 ± 14.20	45.21 ± 26.13
52	22.59 ± 16.56	6.17 ± 6.21	6.66 ± 6.19	4.64 ± 2.89

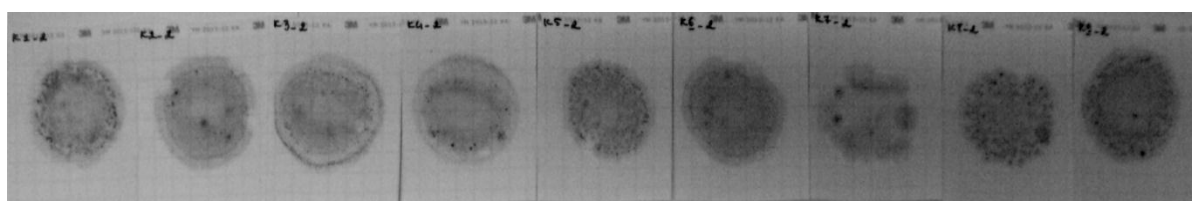
**Table 12:** Yeast growing (cell/cm<sup>2</sup>) on the surface of treated dragon fruits after hot water treatment combined with pH 3.5 (n=9)

Temp (°C)	Time (min)			
	6	9	12	15
46	235.28 ± 74.21	69.62 ± 39.05	129.70 ± 39.05	140.38 ± 73.03
48	86.94 ± 21.58	138.37 ± 73.24	151.36 ± 52.19	140.97 ± 52.98
50	66.10 ± 99.28	530.06 ± 183.83	418.23 ± 129.74	575.29 ± 177.01
52	323.53 ± 16.56	213.55 ± 130.69	198.74 ± 70.62	291.75 ± 173.79

Hot water treatment combined with pH of 3.5 delayed mold growing, but did not change the total number of yeast cells. The untreated control had a total of  $244.66 \pm 15.79$  cfu/cm<sup>2</sup> for the molds and  $489.04 \pm 78.30$  cell/cm<sup>2</sup> yeast. There was a significant difference in the effects of treatment temperature at 52°C for 9-15 minutes on the total number of molds present on the fruit surface.



**Figure 2:** Molds and yeasts present in control samples



**Figure 3:** Molds and yeasts present in treated samples with hot water and pH 3.5

## 4 CONCLUSIONS

The hot water treatment temperature and time combined with low pH adjustment (pH 3.5) significantly ( $p < 0.05$ ) affected the physiological and biochemical characteristics of the dragon fruits. The fruits treated in water with pH 3.5 at 50°C for 12 minutes had higher total acidity and Vitamin C than those treated in the other treatments. Therefore, the physico-chemical treatment using hot water treatment at 50°C for 12 minutes combined with pH 3.5 was tested and successfully applied as a commercial pilot in the dragon fruit pack-house of the Hoang Phat Co. Ltd in Long An province.



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